

09/15/99
JCS586 U.S. PTO

Please type a plus sign (+) inside this box ☐

Attorney Docket **P1101P2**
PATENT

A. Savelli

CERTIFICATION UNDER 37 CFR 1.10

EL 141 796 136 US: Express Mail Number September 15, 1999: Date of Deposit

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Ann Savelli
Ann Savelli

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

JCS49 U.S. PTO
09/396710
09/15/99

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

**Avi J. Ashkenazi
Anan Chuntharapai
K. Jin Kim**

Title: **APO-2 RECEPTOR ANTIBODIES**

1. Type of Application

- [] This application is for an original, non-provisional application.
- [] This is a non-provisional application claiming priority to provisional application no. __, filed __, the entire disclosure of which is hereby incorporated by reference.
- [x] This is a [x] continuation-in-part [] continuation [] divisional application claiming priority to application Serial Number 09/096,637, filed June 12, 1998, and Serial Number 09/020,746, filed February 8, 1998, the entire disclosures of which are hereby incorporated by reference.

**2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b)
(Non-provisional)**

87 pages of specification
2 pages of claims
1 page(s) of abstract
22 sheet(s) of drawings
[] formal [x] informal

09396710-091599

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)
☒ An executed declaration of the inventor(s) [] is enclosed [x] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)
☐ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)
☐ A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)
☒ An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [x] will follow.

(for cont./div.)
☐ The prior application is assigned of record to Genentech, Inc.

5. Amendments (for continuation and divisional applications)

☐ Cancel in this application original claims __ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☐ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

☐ Amend the specification by inserting before the first line the sentence:

--This is a

- ☐ non-provisional application
- ☐ continuation
- ☐ divisional
- ☐ continuation-in-part

of co-pending application(s)

☐ Serial No. _ filed on __, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --

☐ International Application _ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

☐ provisional application No. _ filed __, the entire disclosure of which is hereby incorporated by

66260" 0T 296660

reference and to which application(s) priority is claimed under 35 USC §119.--.

6. Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$760.00
Total Claims	13	- 20 =	0	X \$18.00	\$0.00
Independent Claims	2	- 3 =	0	X \$78.00	\$0.00
Multiple dependent claim(s), if any				+ \$260.00	\$0.00
Filing Fee Calculation					\$760.00

7. Method of Payment of Fees

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$760.00. **A duplicate copy of this transmittal is enclosed.**

8. Authorization to Charge Additional Fees

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

9. Additional Papers Enclosed

- ☐ Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- ☐ Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- ☐ A new Power of Attorney or authorization of agent.
- ☐ Other:

10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

- _____ A petition, fee and/or response has been filed to extend the term in the pending prior application until
- _____ A copy of the petition for extension of time in the **prior** application is attached.

07-0630-07-296660

11. Correspondence Address:

X Address all future communications to:

GENENTECH, INC.
Attn: Diane L. Marschang
1 DNA Way
South San Francisco, CA 94080-4990
(650) 225-1000

Respectfully submitted,
GENENTECH, INC.

Date: September 15, 1999

By: *Diane L. Marschang*
Diane L. Marschang
Reg. No. 35,600

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1000
Fax: (650) 952-9881

650 225 1000

10

Apo-2 Receptor AntibodiesRELATED APPLICATIONS

15

This application is a continuation-in-part application of Application Serial No. 09/096,637 filed June 12, 1998 and a continuation-in-part application of Application Serial No. 09/020,746 filed February 8, 1998, which is a continuation-in-part application of Application Serial No. 08/857,216 filed May 15, 1997, the contents of which are hereby incorporated by reference.

20

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2" and to anti-Apo-2 antibodies.

25

BACKGROUND OF THE INVENTIONApoptosis or "Programmed Cell Death"

30

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)].

35

40

45

Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia,

5 myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

10 Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

TNF Family of Cytokines

25 Various molecules, such as tumor necrosis factor- α ("TNF- α "), tumor necrosis factor- β ("TNF- β " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); WO 97/01633 published January 16, 1997]. Among these molecules, TNF- α , TNF- β , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- α and TNF- β have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- α is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on

5 Programmed Cell Death, Abstr. No. 10, (1995)].

10 Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., 15 supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- α [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

TNF Family of Receptors

20 Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); 25 Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The 35 extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. The cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

40 The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four

5 cysteine-rich domains (CRDs) designated 1 through 4, starting from the
NH₂-terminus. Each CRD is about 40 amino acids long and contains 4 to 6
cysteine residues at positions which are well conserved [Schall et al.,
supra; Loetscher et al., supra; Smith et al., supra; Nophar et al.,
supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of
10 the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2-
amino acids from about 54 to about 97; CRD3- amino acids from about 98
to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2,
CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about
55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4-
15 amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435
(1993)]. The potential role of the CRDs in ligand binding is also
described by Banner et al., supra.

20 A similar repetitive pattern of CRDs exists in several other
cell-surface proteins, including the p75 nerve growth factor receptor
(NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature,
325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J.,
8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063
(1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al.,
supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2
25 proteins of the Shope and myxoma poxviruses [Upton et al., Virology,
160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335
(1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of
these sequences indicates that the positions of the cysteine residues
are well conserved. These receptors are sometimes collectively
30 referred to as members of the TNF/NGF receptor superfamily. Recent
studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et
al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid
insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem.,
266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H.
35 and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of
about 60 amino acids, between its CRD4 and transmembrane region, which
is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol.,
41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973
(1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region
40 is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an
apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh

5 et al., supra]. Expression of the Apo-1 antigen has also been reported
to be down-regulated along with that of TNFR1 when cells are treated
with either TNF- α or anti-Apo-1 mouse monoclonal antibody [Krammer et
al., supra; Nagata et al., supra]. Accordingly, some investigators have
10 hypothesized that cell lines that co-express both Apo-1 and TNFR1
receptors may mediate cell killing through common signaling pathways
[Id.].

The TNF family ligands identified to date, with the exception
of lymphotoxin- α , are type II transmembrane proteins, whose C-terminus
is extracellular. In contrast, the receptors in the TNF receptor (TNFR)
15 family identified to date are type I transmembrane proteins. In both
the TNF ligand and receptor families, however, homology identified
between family members has been found mainly in the extracellular domain
("ECD"). Several of the TNF family cytokines, including TNF- α , Apo-1
ligand and CD40 ligand, are cleaved proteolytically at the cell surface;
20 the resulting protein in each case typically forms a homotrimeric
molecule that functions as a soluble cytokine. TNF receptor family
proteins are also usually cleaved proteolytically to release soluble
receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been
25 identified. In Marsters et al., Curr. Biol., 6:750 (1996),
investigators describe a full length native sequence human polypeptide,
called Apo-3, which exhibits similarity to the TNFR family in its
extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that
it contains a cytoplasmic death domain sequence [see also Marsters et
30 al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by
other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science,
274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al.,
Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member
35 referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4
was reported to contain a cytoplasmic death domain capable of engaging
the cell suicide apparatus. Pan et al. disclose that DR4 is believed to
be a receptor for the ligand known as Apo-2 ligand or TRAIL.

The Apoptosis-Inducing Signaling Complex

40 As presently understood, the cell death program contains at
least three important elements - activators, inhibitors, and effectors;

5 in *C. elegans*, these elements are encoded respectively by three genes,
Ced-4, Ced-9 and Ced-3 [Steller, Science, 267:1445 (1995); Chinnaiyan et
al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members,
TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan
and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell;
10 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the
transcription factor, NF-KB [Tartaglia et al., Cell, 74:845-853 (1993);
Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology,
these two receptors share homology in their intracellular domain (ICD)
in an oligomerization interface known as the death domain [Tartaglia et
15 al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found
in several metazoan proteins that regulate apoptosis, namely, the
Drosophila protein, Reaper, and the mammalian proteins referred to as
FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482
(1995)].

20 Upon ligand binding and receptor clustering, TNFR1 and CD95
are believed to recruit FADD into a death-inducing signalling complex.
CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly
via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J.
25 Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al.,
J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD
serves as an adaptor protein which recruits the Ced-3-related protease,
MACH α /FLICE (caspase 8), into the death signalling complex [Boldin et
al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)].
Caspase 8 appears to be the trigger that sets off a cascade of apoptotic
30 proteases, including the interleukin-1 β converting enzyme (ICE; caspase
1) and CPP32/Yama (caspase 3), which may execute some critical aspects
of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves
the activity of members of a family of cysteine proteases related to the
35 *C. elegans* cell death gene, ced-3, and to the mammalian IL-1-converting
enzyme, ICE (caspase 1). The activity of caspase 1 and 3 can be
inhibited by the product of the cowpox virus gene, crmA [Ray et al.,
Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)].
Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell
40 death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol.
Chem., 270:3255-3260 (1995)].

5 As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40
modulate the expression of proinflammatory and costimulatory cytokines,
cytokine receptors, and cell adhesion molecules through activation of
the transcription factor, NF-KB [Tewari et al., Curr. Op. Genet.
10 Develop., 6:39-44 (1996)]. NF-KB is the prototype of a family of
dimeric transcription factors whose subunits contain conserved Rel
regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann.
Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-KB is
15 complexed with members of the IKB inhibitor family; upon inactivation of
the IKB in response to certain stimuli, released NF-KB translocates to
the nucleus where it binds to specific DNA sequences and activates gene
transcription.

For a review of the TNF family of cytokines and their
receptors, see Gruss and Dower, supra.

20 SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel
polypeptides, designated in the present application as "Apo-2." It is
believed that Apo-2 is a member of the TNFR family; full-length native
sequence human Apo-2 polypeptide exhibits some similarities to some
25 known TNFRs, including a cytoplasmic death domain region. Full-length
native sequence human Apo-2 also exhibits similarity to the TNFR family
in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been
found to be capable of triggering caspase-dependent apoptosis and
activating NF-KB. Applicants surprisingly found that the soluble
30 extracellular domain of Apo-2 binds Apo-2 ligand ("Apo-2L") and can
inhibit Apo-2 ligand function. The crystal structure of the complex
formed between Apo-2 ligand and an extracellular domain sequence of Apo-
2 is described herein. It is presently believed that Apo-2 ligand can
signal via at least two different receptors, DR4 and the newly described
35 Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2
polypeptide. In particular, the invention provides isolated native
sequence Apo-2 polypeptide, which in one embodiment, includes an amino
acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1).
40 In other embodiments, the isolated Apo-2 polypeptide comprises at least

5 about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-2 polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

10 In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

15 In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

20 In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

25 In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

30 (a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

35 (b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

40 (c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e.,

5 nucleotides 1109-1111 through 1310-1312), inclusive; or
(e) a sequence corresponding to the sequence of (a), (b), (c)
or (d) within the scope of degeneracy of the genetic code. The isolated
nucleic acid may comprise the Apo-2 polypeptide cDNA insert of the
vector deposited as ATCC 209021 which includes the nucleotide sequence
10 encoding Apo-2 polypeptide.

In a further embodiment, the invention provides a vector
comprising the nucleic acid molecule encoding the Apo-2 polypeptide or
particular domain of Apo-2. A host cell comprising the vector or the
nucleic acid molecule is also provided. A method of producing Apo-2 is
15 further provided.

In another embodiment, the invention provides an antibody
which specifically binds to Apo-2. The antibody may be an agonistic,
antagonistic, neutralizing, or blocking antibody. Dimeric molecules, in
particular homodimeric molecules, comprising Apo-2 antibody are also
provided.

In another embodiment, the invention provides non-human,
transgenic or knock-out animals.

A further embodiment of the invention provides articles of
manufacture and kits that include Apo-2 or Apo-2 antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence
human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native
sequence human Apo-2 - the putative signal sequence is underlined, the
putative transmembrane domain is boxed, and the putative death domain
sequence is dash underlined. The cysteines of the two cysteine-rich
domains are individually underlined.

Figure 2B shows an alignment and comparison of the death
domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1,
and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential
for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L.
Supernatants from mock-transfected 293 cells or from 293 cells
transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-
His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag
conjugated or Nickel conjugated agarose beads. The precipitated

5 proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based
10 plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed
15 for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF-KB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-KB activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor
30 expressed in human 9D cells.

Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.

Figure 9 is a bar diagram showing percent (%) apoptosis, as
35 compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.

Figure 10 is a bar diagram illustrating the ability of Apo-2 antibody 3F11.39.7 to block the apoptosis induced by Apo-2L in 9D cells.

Figure 11 is a graph showing results of an ELISA testing
40 binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.

5 Figure 12 provides a table identifying Apo-2 antibodies, 2B3.7.1 ("2B3"); 3F11.39.7 ("3F11"); 4B9.23.6 ("4B9"); 5C7.9.1 ("5C7"); 3H1.18.10 ("3H1"); 3H3.14.5 ("3H3"); 3D5.1.10 ("3D5"); 3C9.8.6 ("3C9"); 4H10.14.10 ("4H10"), as well as various properties and activities identified with each respective antibody.

10 Figure 13 shows the binding affinities of Apo-2 antibodies 3F11 and 3H3, to Apo-2-IgG, as determined in a KinExA™ assay. Binding affinities, e.g., of DR4 and Apo-2 immunoadhesins to Apo-2 ligand are shown for comparison.

15 Figure 14 shows apoptotic activity of Apo-2 antibodies, 3D5; 3C9; 5C7; 3H3; 3F11; 4B9; 2B3; and 3H1, on SKMES tumor cells in the presence of goat anti-mouse IgG Fc.

Figure 15A shows apoptotic activity of Apo-2 antibodies, 3F11 and 3H3, on Colo205 tumor cells in the presence or absence of goat anti-mouse IgG Fc.

20 Figure 15B shows apoptotic activity of Apo-2 antibodies, 3F11 and 3H3, on glioma cells in the presence or absence of goat anti-mouse IgG Fc.

25 Figure 16 shows graphs illustrating results of an ELISA testing binding of Apo-2 antibodies, 3D5; 3F11; 3H1; and 3H3, to Apo-2 and to other known Apo-2 ligand receptors referred to as DR4 and DcR1.

Figure 17 shows the results of an ELISA testing for blocking activity of Apo-2 antibodies, 3F11; 3H3; 2B3; 4B9; 5C7; 3H1; 3D5; 3C9; and 4H10.

30 Figure 18 shows the effects of Apo-2 antibodies, 3F11 and 3H3, on the growth of Colo205 tumors in athymic nude mice, as measured by tumor volume.

Figure 19 shows data collection and refinement statistics for the x-ray crystal structure of Apo-2 (DR5) bound to Apo-2 ligand.

35 Figure 20 shows the Apo-2L/Apo-2 complex. The Apo-2L trimer is drawn as ribbon and the three receptors are rendered as tubes. The disordered loop (residues 132-143) in Apo-2L is rendered as small spheres. The bound zinc atom and the bound chloride ion pink are shown as small spheres. Beta strands and relevant loops are labeled. (A) Side view. In this orientation, the membrane of the receptor-containing cell is at the bottom of the figure. (B) View down the three-fold axis of the complex, perpendicular to (A).

40 Figure 21 shows an open-book view of the Apo-2L/Apo-2

5 interface. Apo-2L and one receptor are rendered as space filling models, while the other two receptors are shown as tubes. Residues in the interface are colored by % of buried accessible surface area upon complex formation (1-25%, very light grey; 25-50%, light grey; 50-75%, grey; 75-100%, dark grey). The interface divides into two patches, A and B (labeled). A probe size of 1.4 Angstrom was used to calculate the accessible surface area.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

15 The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

20 A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a

5 form of Apo-2 which is essentially free of the transmembrane and
cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than
1% of such transmembrane and/or cytoplasmic domains and preferably, will
have less than 0.5% of such domains. Optionally, Apo-2 ECD will
10 comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino
acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1). The Apo-2 extracellular
domain sequence may also comprise amino acid residues 1 to 130 of Fig. 1
(SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined
below having at least about 80% amino acid sequence identity with the
15 Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID
NO:1) for a full-length native sequence human Apo-2 or with the deduced
amino acid sequences for an extracellular domain or death domain of Apo-
2 identified herein. Such Apo-2 variants include, for instance, Apo-2
polypeptides wherein one or more amino acid residues are added, or
20 deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID
NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino
acid sequence identity, more preferably at least about 90% amino acid
sequence identity, and even more preferably at least about 95% amino
acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID
25 NO:1) or with the extracellular domain or death domain sequences of Apo-
2.

"Percent (%) amino acid sequence identity" with respect to
the sequences identified herein is defined as the percentage of amino
acid residues in a candidate sequence that are identical with the
30 amino acid residues in the Apo-2 sequence, after aligning the
sequences and introducing gaps, if necessary, to achieve the maximum
percent sequence identity, and not considering any conservative
substitutions as part of the sequence identity. Alignment for
purposes of determining percent amino acid sequence identity can be
35 achieved in various ways that are within the skill in the art can
determine appropriate parameters for measuring alignment, including
assigning algorithms needed to achieve maximal alignment over the
full-length sequences being compared. For purposes herein, percent
amino acid identity values can be obtained using the sequence
40 comparison computer program, ALIGN-2, which was authored by Genentech,
Inc. and the source code of which has been filed with user
documentation in the US Copyright Office, Washington, DC, 20559,

5 registered under the US Copyright Registration No. TXU510087. The
ALIGN-2 program is publicly available through Genentech, Inc., South
San Francisco, CA. All sequence comparison parameters are set by the
ALIGN-2 program and do not vary.

10 The term "epitope tagged" when used herein refers to a
chimeric polypeptide comprising Apo-2, or a domain sequence thereof,
fused to a "tag polypeptide". The tag polypeptide has enough residues
to provide an epitope against which an antibody can be made, yet is
short enough such that it does not interfere with activity of the Apo-2.
15 The tag polypeptide preferably also is fairly unique so that the
antibody does not substantially cross-react with other epitopes.
Suitable tag polypeptides generally have at least six amino acid
residues and usually between about 8 to about 50 amino acid residues
(preferably, between about 10 to about 20 residues).

20 "Isolated," when used to describe the various polypeptides
disclosed herein, means polypeptide that has been identified and
separated and/or recovered from a component of its natural environment.
Contaminant components of its natural environment are materials that
would typically interfere with diagnostic or therapeutic uses for the
polypeptide, and may include enzymes, hormones, and other proteinaceous
25 or non-proteinaceous solutes. In preferred embodiments, the polypeptide
will be purified (1) to a degree sufficient to obtain at least 15
residues of N-terminal or internal amino acid sequence by use of a
spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-
reducing or reducing conditions using Coomassie blue or, preferably,
30 silver stain. Isolated polypeptide includes polypeptide *in situ* within
recombinant cells, since at least one component of the Apo-2 natural
environment will not be present. Ordinarily, however, isolated
polypeptide will be prepared by at least one purification step.

35 An "isolated" Apo-2 nucleic acid molecule is a nucleic acid
molecule that is identified and separated from at least one contaminant
nucleic acid molecule with which it is ordinarily associated in the
natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic
acid molecule is other than in the form or setting in which it is found
in nature. Isolated Apo-2 nucleic acid molecules therefore are
40 distinguished from the Apo-2 nucleic acid molecule as it exists in
natural cells. However, an isolated Apo-2 nucleic acid molecule
includes Apo-2 nucleic acid molecules contained in cells that ordinarily

5 express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for
10 prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example,
15 DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If
20 such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2
25 antibody compositions with polypepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may
30 be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a
35 single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including
40

5 hypervariable) domain of an anti-Apo-2 antibody with a constant domain,
or a light chain with a heavy chain, or a chain from one species with a
chain from another species, or fusions with heterologous proteins,
regardless of species of origin or immunoglobulin class or subclass
10 designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv),
so long as they exhibit the desired biological activity. See, e.g. U.S.
Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production
Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York,
1987).

15 Thus, the modifier "monoclonal" indicates the character of
the antibody as being obtained from a substantially homogeneous
population of antibodies, and is not to be construed as requiring
production of the antibody by any particular method. For example, the
monoclonal antibodies to be used in accordance with the present
invention may be made by the hybridoma method first described by Kohler
20 and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA
methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal
antibodies" may also be isolated from phage libraries generated using
the techniques described in McCafferty et al., Nature, 348:552-554
(1990), for example.

25 "Humanized" forms of non-human (e.g. murine) antibodies are
specific chimeric immunoglobulins, immunoglobulin chains, or fragments
thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding
subsequences of antibodies) which contain minimal sequence derived from
non-human immunoglobulin. For the most part, humanized antibodies are
30 human immunoglobulins (recipient antibody) in which residues from a
complementary determining region (CDR) of the recipient are replaced by
residues from a CDR of a non-human species (donor antibody) such as
mouse, rat, or rabbit having the desired specificity, affinity, and
capacity. In some instances, Fv framework region (FR) residues of the
35 human immunoglobulin are replaced by corresponding non-human residues.
Furthermore, the humanized antibody may comprise residues which are
found neither in the recipient antibody nor in the imported CDR or
framework sequences. These modifications are made to further refine and
optimize antibody performance. In general, the humanized antibody will
40 comprise substantially all of at least one, and typically two, variable
domains, in which all or substantially all of the CDR regions correspond
to those of a non-human immunoglobulin and all or substantially all of

5 the FR regions are those of a human immunoglobulin consensus sequence.
The humanized antibody optimally also will comprise at least a portion
of an immunoglobulin constant region or domain (Fc), typically that of a
human immunoglobulin.

10 The term "cytotoxic agent" as used herein refers to a substance
that inhibits or prevents the function of cells and/or causes
destruction of cells. The term is intended to include radioactive
isotopes (e.g. I¹³¹, I¹²⁵, Y⁹⁰ and Re¹⁸⁶), chemotherapeutic agents, and
toxins such as enzymatically active toxins of bacterial, fungal, plant
or animal origin, or variants and/or fragments thereof.

15 A "chemotherapeutic agent" is a chemical compound useful in the
treatment of cancer. Examples of chemotherapeutic agents include
adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine
arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin,
taxoids, e.g. paclitaxel (TAXOLTM, Bristol-Myers Squibb Oncology,
20 Princeton, NJ), and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony,
Rnace), toxotere, methotrexate, cisplatin, melphalan, vinblastine,
bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone,
vincristine, vinorelbine, carboplatin, teniposide, daunomycin,
carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see
25 U.S. Pat. No. 4,675,187), melphalan and other related nitrogen
mustards. Also included in this definition are hormonal agents that
act to regulate or inhibit hormone action on tumors such as tamoxifen
and onapristone.

30 "Biologically active" and "desired biological activity" with
respect to an Apo-2 polypeptide for the purposes herein means (1) having
the ability to modulate apoptosis (either in an agonistic or stimulating
manner or in an antagonistic or blocking manner) in at least one type of
mammalian cell *in vivo* or *ex vivo*; (2) having the ability to bind Apo-2
ligand; or (3) having the ability to modulate Apo-2 ligand signaling and
35 Apo-2 ligand activity.

The terms "apoptosis" and "apoptotic activity" are used in a
broad sense and refer to the orderly or controlled form of cell death in
mammals that is typically accompanied by one or more characteristic cell
changes, including condensation of cytoplasm, loss of plasma membrane
40 microvilli, segmentation of the nucleus, degradation of chromosomal DNA
or loss of mitochondrial function. This activity can be determined and

5 measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

10 The terms "cancer", "cancerous" or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous
15 cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioma, neuroblastoma, cervical cancer, ovarian cancer, lung cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, glioblastoma, endometrial carcinoma, salivary gland carcinoma, kidney cancer, colorectal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

20 The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats.
25 In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

30 The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that
35 Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2 and Apo-2 variants, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

40 A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing

5 Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

10 The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained
15 from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al.,
20 Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].
25

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ³²P-labeled ATP, biotinylation or enzyme labeling.
30 Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.
35

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if
40 necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into

5 cDNA.

10 Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

15 Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

20 Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

35 Once selected Apo-2 variants are produced, they can be

5 contacted with, for instance, Apo-2L, and the interaction, if any, can
be determined. The interaction between the Apo-2 variant and Apo-2L can
be measured by an *in vitro* assay, such as described in the Examples
below. While any number of analytical measurements can be used to
compare activities and properties between a native sequence Apo-2 and an
10 Apo-2 variant, a convenient one for binding is the dissociation constant
 K_d of the complex formed between the Apo-2 variant and Apo-2L as
compared to the K_d for the native sequence Apo-2. Generally, a ≥ 3 -fold
increase or decrease in K_d per substituted residue indicates that the
substituted residue(s) is active in the interaction of the native
15 sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence
suitable for mutagenesis would include sites within the extracellular
domain, and particularly, within one or both of the cysteine-rich
domains. Such variations can be accomplished using the methods
described above.

As described in the Examples below, the crystal structure of
the complex between Apo-2 ligand and an extracellular domain sequence of
Apo-2 receptor has been determined. Apo-2 resembles TNFR1 in overall
structure with relatively little defined secondary structure. It is
tethered into an elongated shape by a series of seven disulfide bridges,
25 six of which are found in subdomains of Apo-2 (residues 43-84 and 85-
130, respectively; see Fig. 1, SEQ ID NO:1) that correspond structurally
to the second and third CRDs of the TNFR1 receptor. The first disulfide
bridge of Apo-2, between residues 28 and 41 (Fig. 1; SEQ ID NO:1),
corresponds to the last disulfide bridge (between Cys 33 and Cys 52) in
30 CRD1 of TNFR1, while the first 21 residues of Apo-2 are disordered.
Thus, Apo-2 residues 1-42, 43-85, and 86-130 (Fig. 1; SEQ ID NO:1) form
analogous subdomains to CRD1, CRD2 and CRD3 of TNFR1. The three copies
of Apo-2 in the complex are very similar to each other, with the
exception of the C-terminal portion of CRD3 (residues 104-130; Fig. 1;
35 SEQ ID NO:1), which exhibits a rigid-body variation in orientation. The
two loops that form most of the contacts with the ligand have very
similar conformations in all three copies.

Unlike the TNF-beta-TNFR1 complex, where the C-terminal
subdomain of the receptor was disordered, the C-terminal residues of
40 Apo-2 are well ordered up to residue 128 in one copy (the "R" chain)
and up to residue 130 in the other two (the "S" and "T" chains).

5 Residue 130 is predicted to be the final extracellular residue before
the putative single transmembrane helix connecting the receptor to its
intracellular death domain. In the complex as described in Example 21
below, the C-termini of the receptors form a triangle approximately 50
Å on a side. Intriguingly, this same spacing is also found for the
10 receptor binding sites on the TRAF-2 trimer, which is known to
interact with the intracellular portions of some TNFR family members
(albeit not with DR4 or Apo-2) (Park et al., Nature, 398:533-538
(1999); McWhirter et al., Proc. Natl. Acad. Sci., 96:8408-8413
(1999)). This suggests that the observed extracellular geometry could
15 be propagated through the rigid transmembrane helix to the death
domains, and that this spacing may be important for proper triggering
of the intracellular apoptotic cascade.

The interface of the Apo-2 ligand/Apo-2 complex is divided into
two patches- patch A and patch B. The dominant characteristic of
20 patch B in the Apo-2L/Apo-2 interface is the interaction between Tyr
216 of Apo-2L (using the numbering of the amino acid sequence for Apo-
2L provided in Pitti et al., J. Biol. Chem., 271:12687-12690 (1996))
and the 50s loop of the Apo-2 receptor. Residue Tyr 216 is conserved
in many of the TNF superfamily ligands (including TNF-alpha, TNF-beta,
25 FasL and OPGL), while other members have a similar large hydrophobic
residue at this position. Mutagenesis studies on TNF-alpha, TNF-beta,
FasL and Apo-2L have all shown that this residue is critical for
binding (Schneider et al., J. Biol. Chem., 272:18827-18833 (1997); Goh
et al., Protein Eng., 4:785-791 (1991); Yamagishi et al., Protein
30 Eng., 3:713-719 (1990); Van Ostade et al., Protein Eng., 7:5-22
(1990); Hymowitz et al., personal communication). The interactions of
the tyrosine side chain are conserved between the Apo-2L/Apo-2 and
TNF-beta-TNFR1 complexes. Moreover, the backbone conformation of the
50s loop of the receptor, which forms the binding pocket for the side
35 chain, is virtually identical between Apo-2 and the TNFR1 (rmsd of
only 0.35 between the C-alpha atoms of residues 51 to 62).
Additionally, the length of this loop is conserved among the different
TNF receptor superfamily members. It is believed that this loop may
function as a general hydrophobic binding patch interacting with
40 conserved hydrophobic features on the ligand which may help properly
orient the upper part of the receptor for more specific contacts
mediated by CRD3.

5 In contrast to the conserved interactions in patch B, patch
A near the bottom of the interface involves interactions made by the
90s loop on CRD3 of Apo-2, which has a completely different
conformation than the corresponding loop in the TNFR1.

10 In patch B, it is believed that the 50s loop of the receptor
and Apo-2 ligand residue 216 provide a hydrophobic patch generally
important for binding, whereas in patch A, the receptor 90s loop and
the Apo-2 ligand residue at or near position 205 control the
specificity and cross-reactivity. Based on the structure identified
by Applicants, the 50s loop and the 90s loop of the Apo-2 receptor are
15 expected to carry most of the ligand-binding determinants.
Optionally, the Apo-2 variants of the invention will retain or possess
native sequence amino acid residues (as provided in Figure 1) in the
50s and/or 90s loop. It is contemplated that Apo-2 may be mutated (by
insertion, deletion, and/or substitution) in certain aspects which
20 will affect ligand binding. The histidine and phenylalanine residues
at positions 53 and 59, respectively, of the Apo-2 sequence are both
relatively large residues. These two residues are believed to contact
residues Asp218 and Ser159 of the Apo-2 ligand; thus introducing
larger side chains at the 53 and 59 positions of the Apo-2 sequence
25 may adversely affect Apo-2L affinity for Apo-2 (but improve affinity
for DR4). Similar mutations may be accomplished at positions 62, 65,
and/or 104 of the Apo-2 sequence. Mutations in any one or all of the
Apo-2 residues at positions 50 through 62 may affect binding of amino
acid residues at or near residue Tyr216 of the Apo-2L sequence, while
30 mutations in any one or all of the Apo-2 residues at positions 65
through 69, 90 through 104, 108, 111, or 122 may affect binding of
amino acid residues at or near residue Gln205 of Apo-2 ligand. It is
contemplated that mutations at such residue(s) may enhance or decrease
interactions (such as binding) with Apo-2 ligand.

35 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2
may be inserted into a replicable vector for further cloning
(amplification of the DNA) or for expression. Various vectors are
publicly available. The vector components generally include, but are
40 not limited to, one or more of the following: a signal sequence, an
origin of replication, one or more marker genes, an enhancer element, a
promoter, and a transcription termination sequence, each of which is

5 described below.

(i) Signal Sequence Component

10 The Apo-2 may be produced recombinantly not only directly,
but also as a fusion polypeptide with a heterologous polypeptide, which
may be a signal sequence or other polypeptide having a specific cleavage
site at the N-terminus of the mature protein or polypeptide. In
general, the signal sequence may be a component of the vector, or it may
be a part of the Apo-2 DNA that is inserted into the vector. The
heterologous signal sequence selected preferably is one that is
recognized and processed (*i.e.*, cleaved by a signal peptidase) by the
15 host cell. The signal sequence may be a prokaryotic signal sequence
selected, for example, from the group of the alkaline phosphatase,
penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast
secretion the signal sequence may be, *e.g.*, the yeast invertase leader,
alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor
20 leaders, the latter described in U.S. Pat. No. 5,010,182), or acid
phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179
published 4 April 1990), or the signal described in WO 90/13646
published 15 November 1990. In mammalian cell expression the native
Apo-2 presequence that normally directs insertion of Apo-2 in the cell
25 membrane of human cells *in vivo* is satisfactory, although other
mammalian signal sequences may be used to direct secretion of the
protein, such as signal sequences from secreted polypeptides of the same
or related species, as well as viral secretory leaders, for example, the
herpes simplex glycoprotein D signal.

30 The DNA for such precursor region is preferably ligated in
reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid
sequence that enables the vector to replicate in one or more selected
35 host cells. Generally, in cloning vectors this sequence is one that
enables the vector to replicate independently of the host chromosomal
DNA, and includes origins of replication or autonomously replicating
sequences. Such sequences are well known for a variety of bacteria,
yeast, and viruses. The origin of replication from the plasmid pBR322
40 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is
suitable for yeast, and various viral origins (SV40, polyoma,

5 adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

10 Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

15 DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

25 Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

35 One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes

5 under eukaryotic control to convey resistance to the appropriate drug
G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin,
respectively.

10 Another example of suitable selectable markers for mammalian
cells are those that enable the identification of cells competent to
take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The
mammalian cell transformants are placed under selection pressure that
only the transformants are uniquely adapted to survive by virtue of
having taken up the marker. Selection pressure is imposed by culturing
the transformants under conditions in which the concentration of
15 selection agent in the medium is successively changed, thereby leading
to amplification of both the selection gene and the DNA that encodes
Apo-2. Amplification is the process by which genes in greater demand
for the production of a protein critical for growth are reiterated in
tandem within the chromosomes of successive generations of recombinant
20 cells. Increased quantities of Apo-2 are synthesized from the amplified
DNA. Other examples of amplifiable genes include metallothionein-I and
-II, adenosine deaminase, and ornithine decarboxylase.

25 Cells transformed with the DHFR selection gene may first be
identified by culturing all of the transformants in a culture medium
that contains methotrexate (Mtx), a competitive antagonist of DHFR. An
appropriate host cell when wild-type DHFR is employed is the Chinese
hamster ovary (CHO) cell line deficient in DHFR activity, prepared and
propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA,
77:4216 (1980). The transformed cells are then exposed to increased
30 levels of methotrexate. This leads to the synthesis of multiple copies
of the DHFR gene, and, concomitantly, multiple copies of other DNA
comprising the expression vectors, such as the DNA encoding Apo-2. This
amplification technique can be used with any otherwise suitable host,
e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous
35 DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx
is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that
contain endogenous DHFR) transformed or co-transformed with DNA
sequences encoding Apo-2, wild-type DHFR protein, and another selectable
40 marker such as aminoglycoside 3'-phosphotransferase (APH) can be
selected by cell growth in medium containing a selection agent for the
selectable marker such as an aminoglycosidic antibiotic, e.g.,

5 kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

10 A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 15 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

20 In addition, vectors derived from the 1.6 μ m circular plasmid pKDI can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

25 (iv) Promoter Component

30 Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the 35 40

5 native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a
10 tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker
15 operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the
20 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast
30 hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase,
35 glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth
40 conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with

5 nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate
dehydrogenase, and enzymes responsible for maltose and galactose
utilization. Suitable vectors and promoters for use in yeast expression
are further described in EP 73,657. Yeast enhancers also are
advantageously used with yeast promoters.

10 Apo-2 transcription from vectors in mammalian host cells is
controlled, for example, by promoters obtained from the genomes of
viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5
July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus,
avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus
15 and most preferably Simian Virus 40 (SV40), from heterologous mammalian
promoters, e.g., the actin promoter or an immunoglobulin promoter, from
heat-shock promoters, and from the promoter normally associated with the
Apo-2 sequence, provided such promoters are compatible with the host
cell systems.

20 The early and late promoters of the SV40 virus are
conveniently obtained as an SV40 restriction fragment that also contains
the SV40 viral origin of replication [Fiers et al., Nature, 273:113
(1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et
al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate
25 early promoter of the human cytomegalovirus is conveniently obtained as
a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360
(1982)]. A system for expressing DNA in mammalian hosts using the bovine
papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446.
A modification of this system is described in U.S. Patent No. 4,601,978
30 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA
encoding immune interferon in monkey cells; Reyes et al., Nature,
297:598-601 (1982) on expression of human β -interferon cDNA in mouse
cells under the control of a thymidine kinase promoter from herpes
simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170
35 (1982) on expression of the human interferon gene in cultured mouse and
rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-
6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey
kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells,
HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long
40 terminal repeat as a promoter].

5 (v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12

5 strain 294 (ATCC 31,446) and successful transformants selected by
ampicillin or tetracycline resistance where appropriate. Plasmids from
the transformants are prepared, analyzed by restriction endonuclease
digestion, and/or sequenced by the method of Messing et al., Nucleic
10 Acids Res., 9:309 (1981) or by the method of Maxim et al., Methods in
Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression
in mammalian cells of DNA encoding Apo-2 may be employed. In general,
transient expression involves the use of an expression vector that is
15 able to replicate efficiently in a host cell, such that the host cell
accumulates many copies of the expression vector and, in turn,
synthesizes high levels of a desired polypeptide encoded by the
expression vector [Sambrook et al., *supra*]. Transient expression
systems, comprising a suitable expression vector and a host cell, allow
20 for the convenient positive identification of polypeptides encoded by
cloned DNAs, as well as for the rapid screening of such polypeptides for
desired biological or physiological properties. Thus, transient
expression systems are particularly useful in the invention for purposes
of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for
adaptation to the synthesis of Apo-2 in recombinant vertebrate cell
culture are described in Gething et al., Nature, 293:620-625 (1981);
Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the
vectors herein are the prokaryote, yeast, or higher eukaryote cells
described above. Suitable prokaryotes for this purpose include but are
not limited to eubacteria, such as Gram-negative or Gram-positive
35 organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E.*
coli, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g.,
Salmonella typhimurium, *Serratia*, e.g., *Serratia marcescans*, and
Shigella, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis*
(e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April
40 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably,
the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as

5 filamentous fungi or yeast are suitable cloning or expression hosts for
Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's
yeast, is the most commonly used among lower eukaryotic host
microorganisms. However, a number of other genera, species, and strains
are commonly available and useful herein.

10 Suitable host cells for the expression of glycosylated Apo-2
are derived from multicellular organisms. Such host cells are capable
of complex processing and glycosylation activities. In principle, any
higher eukaryotic cell culture is workable, whether from vertebrate or
invertebrate culture. Examples of invertebrate cells include plant and
15 insect cells. Numerous baculoviral strains and variants and
corresponding permissive insect host cells from hosts such as *Spodoptera*
frugiperda (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus*
(mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have
been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55
20 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol.
8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature,
315:592-594 (1985)]. A variety of viral strains for transfection are
publicly available, e.g., the L-1 variant of *Autographa californica* NPV
and the Bm-5 strain of *Bombyx mori* NPV.

25 Plant cell cultures of cotton, corn, potato, soybean,
petunia, tomato, and tobacco can be utilized as hosts. Typically, plant
cells are transfected by incubation with certain strains of the
bacterium *Agrobacterium tumefaciens*. During incubation of the plant
cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be
30 transferred to the plant cell host such that it is transfected, and
will, under appropriate conditions, express the Apo-2-encoding DNA. In
addition, regulatory and signal sequences compatible with plant cells
are available, such as the nopaline synthase promoter and
polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen.,
35 1:561 (1982)]. In addition, DNA segments isolated from the upstream
region of the T-DNA 780 gene are capable of activating or increasing
transcription levels of plant-expressible genes in recombinant DNA-
containing plant tissue [EP 321,196 published 21 June 1989].

40 Propagation of vertebrate cells in culture (tissue culture)
is also well known in the art [See, e.g., Tissue Culture, Academic
Press, Kruse and Patterson, editors (1973)]. Examples of useful
mammalian host cell lines are monkey kidney CV1 line transformed by SV40

5 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells
subcloned for growth in suspension culture, Graham et al., J. Gen
Viro., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10);
Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl.
Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol.
10 Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70);
African green monkey kidney cells (VERO-76, ATCC CRL-1587); human
cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK,
ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human
lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065);
15 mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al.,
Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with
the above-described expression or cloning vectors for Apo-2 production
and cultured in conventional nutrient media modified as appropriate for
inducing promoters, selecting transformants, or amplifying the genes
encoding the desired sequences.

Transfection refers to the taking up of an expression vector
by a host cell whether or not any coding sequences are in fact
expressed. Numerous methods of transfection are known to the ordinarily
skilled artisan, for example, CaPO_4 and electroporation. Successful
25 transfection is generally recognized when any indication of the
operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that
the DNA is replicable, either as an extrachromosomal element or by
chromosomal integrant. Depending on the host cell used, transformation
is done using standard techniques appropriate to such cells. The
calcium treatment employing calcium chloride, as described in Sambrook
et al., supra, or electroporation is generally used for prokaryotes or
other cells that contain substantial cell-wall barriers. Infection with
35 *Agrobacterium tumefaciens* is used for transformation of certain plant
cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859
published 29 June 1989. In addition, plants may be transfected using
ultrasound treatment as described in WO 91/00358 published 10 January
1991.

40 For mammalian cells without such cell walls, the calcium
phosphate precipitation method of Graham and van der Eb, Virology,
52:456-457 (1978) is preferred. General aspects of mammalian cell host

5 system transformations have been described in U.S. Pat. No. 4,399,216.
Transformations into yeast are typically carried out according to the
method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et
al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other
10 methods for introducing DNA into cells, such as by nuclear
microinjection, electroporation, bacterial protoplast fusion with intact
cells, or polycations, e.g., polybrene, polyornithine, may also be used.
For various techniques for transforming mammalian cells, see Keown et
al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al.,
Nature, 336:348-352 (1988).

15 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in
suitable media as described generally in Sambrook et al., supra.

20 The mammalian host cells used to produce Apo-2 may be
cultured in a variety of media. Examples of commercially available
media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM",
Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium
("DMEM", Sigma). Any such media may be supplemented as necessary with
hormones and/or other growth factors (such as insulin, transferrin, or
epidermal growth factor), salts (such as sodium chloride, calcium,
25 magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as
adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace
elements (defined as inorganic compounds usually present at final
concentrations in the micromolar range), and glucose or an equivalent
energy source. Any other necessary supplements may also be included at
30 appropriate concentrations that would be known to those skilled in the
art. The culture conditions, such as temperature, pH, and the like, are
those previously used with the host cell selected for expression, and
will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques
35 for maximizing the productivity of mammalian cell cultures can be found
in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed.
(IRL Press, 1991).

The host cells referred to in this disclosure encompass cells
in culture as well as cells that are within a host animal.

40 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a
sample directly, for example, by conventional Southern blotting,

5 Northern blotting to quantitate the transcription of mRNA [Thomas, Proc.
Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis),
or *in situ* hybridization, using an appropriately labeled probe, based on
the sequences provided herein. Various labels may be employed, most
commonly radioisotopes, and particularly ³²P. However, other techniques
10 may also be employed, such as using biotin-modified nucleotides for
introduction into a polynucleotide. The biotin then serves as the site
for binding to avidin or antibodies, which may be labeled with a wide
variety of labels, such as radionucleotides, fluorescers or enzymes.
Alternatively, antibodies may be employed that can recognize specific
15 duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid
duplexes or DNA-protein duplexes. The antibodies in turn may be labeled
and the assay may be carried out where the duplex is bound to a surface,
so that upon the formation of duplex on the surface, the presence of
antibody bound to the duplex can be detected.

20 Gene expression, alternatively, may be measured by
immunological methods, such as immunohistochemical staining of cells or
tissue sections and assay of cell culture or body fluids, to quantitate
directly the expression of gene product. With immunohistochemical
staining techniques, a cell sample is prepared, typically by dehydration
25 and fixation, followed by reaction with labeled antibodies specific for
the gene product coupled, where the labels are usually visually
detectable, such as enzymatic labels, fluorescent labels, or luminescent
labels.

30 Antibodies useful for immunohistochemical staining and/or
assay of sample fluids may be either monoclonal or polyclonal, and may
be prepared in any mammal. Conveniently, the antibodies may be prepared
against a native sequence Apo-2 polypeptide or against a synthetic
peptide based on the DNA sequences provided herein or against exogenous
sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

35 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from
host cell lysates. If the Apo-2 is membrane-bound, it can be released
from the membrane using a suitable detergent solution (e.g. Triton-X
100) or its extracellular domain may be released by enzymatic cleavage.

40 When Apo-2 is produced in a recombinant cell other than one
of human origin, the Apo-2 is free of proteins or polypeptides of human
origin. However, it may be desired to purify Apo-2 from recombinant

5 cell proteins or polypeptides to obtain preparations that are
substantially homogeneous as to Apo-2. As a first step, the culture
medium or lysate may be centrifuged to remove particulate cell debris.
Apo-2 thereafter is purified from contaminant soluble proteins and
polypeptides, with the following procedures being exemplary of suitable
10 purification procedures: by fractionation on an ion-exchange column;
ethanol precipitation; reverse phase HPLC; chromatography on silica or
on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE;
ammonium sulfate precipitation; gel filtration using, for example,
Sephadex G-75; and protein A Sepharose columns to remove contaminants
15 such as IgG.

Apo-2 variants in which residues have been deleted, inserted,
or substituted can be recovered in the same fashion as native sequence
Apo-2, taking account of changes in properties occasioned by the
variation. For example, preparation of an Apo-2 fusion with another
protein or polypeptide, e.g., a bacterial or viral antigen,
immunoglobulin sequence, or receptor sequence, may facilitate
purification; an immunoaffinity column containing antibody to the
sequence can be used to adsorb the fusion polypeptide. Other types of
affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride
(PMSF) also may be useful to inhibit proteolytic degradation during
purification, and antibiotics may be included to prevent the growth of
adventitious contaminants. One skilled in the art will appreciate that
purification methods suitable for native sequence Apo-2 may require
25 modification to account for changes in the character of Apo-2 or its
variants upon expression in recombinant cell culture.

7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope
of this invention. One type of covalent modification of the Apo-2 is
35 introduced into the molecule by reacting targeted amino acid residues of
the Apo-2 with an organic derivatizing agent that is capable of reacting
with selected side chains or the N- or C- terminal residues of the Apo-
2.

Derivatization with bifunctional agents is useful for
40 crosslinking Apo-2 to a water-insoluble support matrix or surface for
use in the method for purifying anti-Apo-2 antibodies, and vice-versa.
Derivatization with one or more bifunctional agents will also be useful

5 for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers
may increase binding avidity and extend half-life of the molecule *in*
vivo. Commonly used crosslinking agents include, e.g., 1,1-bis(diazo-
acetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for
example, esters with 4-azidosalicylic acid, homobifunctional
10 imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-
(succinimidylpropionate), and bifunctional maleimides such as bis-N-
maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-
azidophenyl)dithio]propioimide yield photoactivatable intermediates
that are capable of forming crosslinks in the presence of light.
15 Alternatively, reactive water-insoluble matrices such as cyanogen
bromide-activated carbohydrates and the reactive substrates described in
U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537;
and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutamyl and
20 asparaginy l residues to the corresponding glutamyl and aspartyl
residues, respectively, hydroxylation of proline and lysine,
phosphorylation of hydroxyl groups of seryl or threonyl residues,
methylation of the α -amino groups of lysine, arginine, and histidine
side chains [T.E. Creighton, Proteins: Structure and Molecular
25 Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)],
acetylation of the N-terminal amine, and amidation of any C-terminal
carboxyl group. The modified forms of the residues fall within the
scope of the present invention.

Another type of covalent modification of the Apo-2
30 polypeptide included within the scope of this invention comprises
altering the native glycosylation pattern of the polypeptide. "Altering
the native glycosylation pattern" is intended for purposes herein to
mean deleting one or more carbohydrate moieties found in native sequence
Apo-2, and/or adding one or more glycosylation sites that are not
35 present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or
O-linked. N-linked refers to the attachment of the carbohydrate moiety
to the side chain of an asparagine residue. The tripeptide sequences
asparagine-X-serine and asparagine-X-threonine, where X is any amino
40 acid except proline, are the recognition sequences for enzymatic
attachment of the carbohydrate moiety to the asparagine side chain.

5 Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-
10 hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the
15 addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated
20 that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used,
25 the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These
30 methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by
35 mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylglactosamine), while leaving the
40 polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge

5 et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

10 Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

15 Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

8. Apo-2 Chimeras

20 The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

25 In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2 to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

30 Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide

5 tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397
(1990)]. Once the tag polypeptide has been selected, an antibody
thereto can be generated using the techniques disclosed herein.

10 Generally, epitope-tagged Apo-2 may be constructed and
produced according to the methods described above. Epitope-tagged Apo-2
is also described in the Examples below. Apo-2-tag polypeptide fusions
are preferably constructed by fusing the cDNA sequence encoding the Apo-
2 portion in-frame to the tag polypeptide DNA sequence and expressing
the resultant DNA fusion construct in appropriate host cells.
Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the
15 present invention, nucleic acid encoding the Apo-2 will be fused at its
3' end to nucleic acid encoding the N-terminus of the tag polypeptide,
however 5' fusions are also possible. For example, a polyhistidine
sequence of about 5 to about 10 histidine residues may be fused at the
N- terminus or the C- terminus and used as a purification handle in
20 affinity chromatography.

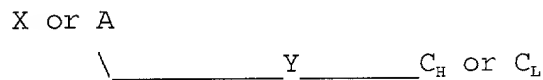
Epitope-tagged Apo-2 can be purified by affinity
chromatography using the anti-tag antibody. The matrix to which the
affinity antibody is attached may include, for instance, agarose,
controlled pore glass or poly(styrenedivinyl)benzene. The epitope-
25 tagged Apo-2 can then be eluted from the affinity column using
techniques known in the art.

In another embodiment, the chimeric molecule comprises an
Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric
molecule may also comprise a particular domain sequence of Apo-2, such
30 as the extracellular domain sequence of native Apo-2 fused to an
immunoglobulin sequence. This includes chimeras in monomeric, homo- or
heteromultimeric, and particularly homo- or heterodimeric, or -
tetrameric forms; optionally, the chimeras may be in dimeric forms or
homodimeric heavy chain forms. Generally, these assembled
35 immunoglobulins will have known unit structures as represented by the
following diagrams.

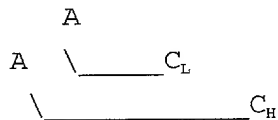
5



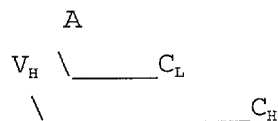
10



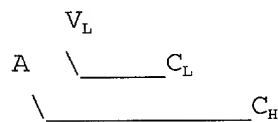
15



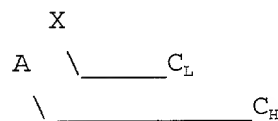
20



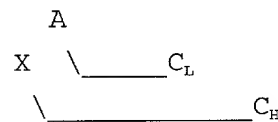
25



30



35



A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same

5 or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

10 monomer: A _____ C_L or C_H

homodimer: A
 \

 /
 A

20 heterodimer: A
 \

 /
 X

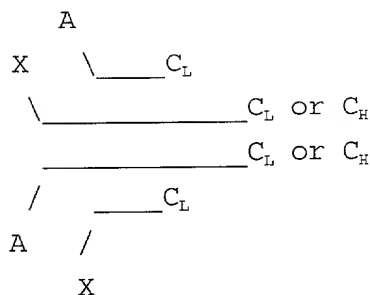
25 homotetramer: A
 A \

 /
 A /
 A

35 heterotetramer: A
 A \

 /
 X /
 X

5 and



15 In the foregoing diagrams, "A" means an Apo-2 sequence or an
Apo-2 sequence fused to a heterologous sequence; X is an additional
agent, which may be the same as A or different, a portion of an
immunoglobulin superfamily member such as a variable region or a
variable region-like domain, including a native or chimeric
immunoglobulin variable region, a toxin such a pseudomonas exotoxin or
ricin, or a sequence functionally binding to another protein, such as
other cytokines (i.e., IL-1, interferon- γ) or cell surface molecules
(i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma
poxviruses), or a polypeptide therapeutic agent not otherwise normally
associated with a constant domain; Y is a linker or another receptor
sequence; and V_L, V_H, C_L and C_H represent light or heavy chain variable
or constant domains of an immunoglobulin. Structures comprising at
least one CRD of an Apo-2 sequence as "A" and another cell-surface
protein having a repetitive pattern of CRDs (such as TNFR) as "X" are
specifically included.

20
25
30 It will be understood that the above diagrams are merely
exemplary of the possible structures of the chimeras of the present
invention, and do not encompass all possibilities. For example, there
might desirably be several different "A"s, "X"s, or "Y"s in any of these
constructs. Also, the heavy or light chain constant domains may be
originated from the same or different immunoglobulins. All possible
permutations of the illustrated and similar structures are all within
the scope of the invention herein.

35
40 In general, the chimeric molecules can be constructed in a
fashion similar to chimeric antibodies in which a variable domain from
an antibody of one species is substituted for the variable domain of

5 another species. See, for example, EP 0 125 023; EP 173,494; Munro,
Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-
608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May
1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855
10 al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature,
337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

15 Alternatively, the chimeric molecules may be constructed as
follows. The DNA including a region encoding the desired sequence, such
as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at
or proximal to the 3' end of the DNA encoding the immunoglobulin-like
domain(s) and at a point at or near the DNA encoding the N-terminal end
of the Apo-2 or TNFR polypeptide (where use of a different leader is
contemplated) or at or proximal to the N-terminal coding region for TNFR
(where the native signal is employed). This DNA fragment then is
20 readily inserted proximal to DNA encoding an immunoglobulin light or
heavy chain constant region and, if necessary, the resulting construct
tailored by deletional mutagenesis. Preferably, the Ig is a human
immunoglobulin when the chimeric molecule is intended for *in vivo*
therapy for humans. DNA encoding immunoglobulin light or heavy chain
constant regions is known or readily available from cDNA libraries or is
25 synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719
(1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al.,
Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc.
Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-
30 288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in
publications concerning the preparation of immunoadhesins.
Immunoadhesins in general, and CD4-Ig fusion molecules specifically are
disclosed in WO 89/02922, published 6 April 1989. Molecules comprising
35 the extracellular portion of CD4, the receptor for human
immunodeficiency virus (HIV), linked to IgG heavy chain constant region
are known in the art and have been found to have a markedly longer half-
life and lower clearance than the soluble extracellular portion of CD4
[Capon et al., *supra*; Byrn et al., Nature, 344:667 (1990)]. The
40 construction of specific chimeric TNFR-IgG molecules is also described
in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991);
Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P

5 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

10 Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically
15 to inhibit apoptosis or NF-KB induction by Apo-2L or by another ligand that Apo-2 binds to.

20 The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

25 The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity
30 purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

35 Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

40 Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a

5 prenatal, e.g., an embryonic stage. A transgene is a DNA which is
integrated into the genome of a cell from which a transgenic animal
develops. In one embodiment, cDNA encoding Apo-2 or an appropriate
sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA
10 sequences used to generate transgenic animals that contain cells which
express DNA encoding Apo-2. Methods for generating transgenic animals,
particularly animals such as mice or rats, have become conventional in
the art and are described, for example, in U.S. Patent Nos. 4,736,866
and 4,870,009. Typically, particular cells would be targeted for Apo-2
15 transgene incorporation with tissue-specific enhancers. Transgenic
animals that include a copy of a transgene encoding Apo-2 introduced
into the germ line of the animal at an embryonic stage can be used to
examine the effect of increased expression of DNA encoding Apo-2. Such
animals can be used as tester animals for reagents thought to confer
20 protection from, for example, pathological conditions associated with
excessive apoptosis. In accordance with this facet of the invention, an
animal is treated with the reagent and a reduced incidence of the
pathological condition, compared to untreated animals bearing the
transgene, would indicate a potential therapeutic intervention for the
25 pathological condition. In another embodiment, transgenic animals that
carry a soluble form of Apo-2 such as an Apo-2 ECD or an immunoglobulin
chimera of such form could be constructed to test the effect of chronic
neutralization of Apo-2L, a ligand of Apo-2.

30 Alternatively, non-human homologues of Apo-2 can be used to
construct an Apo-2 "knock out" animal which has a defective or altered
gene encoding Apo-2 as a result of homologous recombination between the
endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2
introduced into an embryonic cell of the animal. For example, cDNA
encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in
35 accordance with established techniques. A portion of the genomic DNA
encoding Apo-2 can be deleted or replaced with another gene, such as a
gene encoding a selectable marker which can be used to monitor
integration. Typically, several kilobases of unaltered flanking DNA
(both at the 5' and 3' ends) are included in the vector [see e.g.,
40 Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous
recombination vectors]. The vector is introduced into an embryonic stem
cell line (e.g., by electroporation) and cells in which the introduced

5 DNA has homologously recombined with the endogenous DNA are selected
[see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then
injected into a blastocyst of an animal (e.g., a mouse or rat) to form
aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and*
Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed. (IRL,
10 Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted
into a suitable pseudopregnant female foster animal and the embryo
brought to term to create a "knock out" animal. Progeny harboring the
homologously recombined DNA in their germ cells can be identified by
standard techniques and used to breed animals in which all cells of the
15 animal contain the homologously recombined DNA. Knockout animals can be
characterized for instance, for their ability to defend against certain
pathological conditions and for their development of pathological
conditions due to absence of the Apo-2 polypeptide, including for
example, development of tumors.

20 C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies.
Antibodies against Apo-2 may be prepared as follows. Exemplary
antibodies include polyclonal, monoclonal, humanized, bispecific, and
heteroconjugate antibodies.

25 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies.
Methods of preparing polyclonal antibodies are known to the skilled
artisan. Polyclonal antibodies can be raised in a mammal, for example,
by one or more injections of an immunizing agent and, if desired, an
30 adjuvant. Typically, the immunizing agent and/or adjuvant will be
injected in the mammal by multiple subcutaneous or intraperitoneal
injections. The immunizing agent may include the Apo-2 polypeptide or a
fusion protein thereof. An example of a suitable immunizing agent is an
Apo-2-IgG fusion protein, such as an Apo-2 ECD-IgG fusion protein.
35 Cells expressing Apo-2 at their surface may also be employed. It may be
useful to conjugate the immunizing agent to a protein known to be
immunogenic in the mammal being immunized. Examples of such immunogenic
proteins which may be employed include but are not limited to keyhole
limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean
40 trypsin inhibitor. An aggregating agent such as alum may also be
employed to enhance the mammal's immune response. Examples of adjuvants
which may be employed include Freund's complete adjuvant and MPL-TDM

5 adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).
The immunization protocol may be selected by one skilled in the art
without undue experimentation. The mammal can then be bled, and the
serum assayed for antibody titer. If desired, the mammal can be boosted
until the antibody titer increases or plateaus.

10 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal
antibodies. Monoclonal antibodies may be prepared using hybridoma
methods, such as those described by Kohler and Milstein, supra. In a
hybridoma method, a mouse, hamster, or other appropriate host animal, is
15 typically immunized (such as described above) with an immunizing agent
to elicit lymphocytes that produce or are capable of producing
antibodies that will specifically bind to the immunizing agent.
Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2
polypeptide or a fusion protein thereof. An example of a suitable
immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A
specific example of an Apo-2 ECD-IgG immunogen is described in Example 9
below. Cells expressing Apo-2 at their surface may also be employed.
Generally, either peripheral blood lymphocytes ("PBLs") are used if
cells of human origin are desired, or spleen cells or lymph node cells
are used if non-human mammalian sources are desired. The lymphocytes
are then fused with an immortalized cell line using a suitable fusing
agent, such as polyethylene glycol, to form a hybridoma cell [Goding,
25 Monoclonal Antibodies: Principles and Practice, Academic Press, (1986)
pp. 59-103]. Immortalized cell lines are usually transformed mammalian
cells, particularly myeloma cells of rodent, bovine and human origin.
Usually, rat or mouse myeloma cell lines are employed. The hybridoma
cells may be cultured in a suitable culture medium that preferably
contains one or more substances that inhibit the growth or survival of
35 the unfused, immortalized cells. For example, if the parental
transformed cells lack the enzyme hypoxanthine guanine phosphoribosyl
transferase (HGPRT or HPRT), the culture medium for the hybridomas
typically will include hypoxanthine, aminopterin, and thymidine ("HAT
medium"), which substances prevent the growth of HGPRT-deficient cells.

40 Preferred immortalized cell lines are those that fuse
efficiently, support stable high level expression of antibody by the
selected antibody-producing cells, and are sensitive to a medium such as

5 HAT medium. More preferred immortalized cell lines are murine myeloma
lines, which can be obtained, for instance, from the Salk Institute Cell
Distribution Center, San Diego, California and the American Type Culture
Collection, Manassas, Virginia. Human myeloma and mouse-human
heteromyeloma cell lines also have been described for the production of
10 human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984);
Brodeur et al., Monoclonal Antibody Production Techniques and
Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured
can then be assayed for the presence of monoclonal antibodies directed
15 against Apo-2. Preferably, the binding specificity of monoclonal
antibodies produced by the hybridoma cells is determined by
immunoprecipitation or by an *in vitro* binding assay, such as
radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).
Such techniques and assays are known in the art. The binding affinity
20 of the monoclonal antibody can, for example, be determined by the
Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220
(1980).

After the desired hybridoma cells are identified, the clones
may be subcloned by limiting dilution procedures and grown by standard
25 methods [Goding, supra]. Suitable culture media for this purpose
include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640
medium. Alternatively, the hybridoma cells may be grown *in vivo* as
ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be
30 isolated or purified from the culture medium or ascites fluid by
conventional immunoglobulin purification procedures such as, for
example, protein A-Sepharose, hydroxylapatite chromatography, gel
electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA
35 methods, such as those described in U.S. Patent No. 4,816,567. DNA
encoding the monoclonal antibodies of the invention can be readily
isolated and sequenced using conventional procedures (e.g., by using
oligonucleotide probes that are capable of binding specifically to genes
encoding the heavy and light chains of murine antibodies). The
40 hybridoma cells of the invention serve as a preferred source of such
DNA. Once isolated, the DNA may be placed into expression vectors,
which are then transfected into host cells such as simian COS cells,

5 Chinese hamster ovary (CHO) cells, or myeloma cells that do not
otherwise produce immunoglobulin protein, to obtain the synthesis of
monoclonal antibodies in the recombinant host cells. The DNA also may
be modified, for example, by substituting the coding sequence for human
heavy and light chain constant domains in place of the homologous murine
10 sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by
covalently joining to the immunoglobulin coding sequence all or part of
the coding sequence for a non-immunoglobulin polypeptide. Such a non-
immunoglobulin polypeptide can be substituted for the constant domains
of an antibody of the invention, or can be substituted for the variable
15 domains of one antigen-combining site of an antibody of the invention to
create a chimeric bivalent antibody.

As described in the Examples below, anti-Apo-2 monoclonal
antibodies have been prepared. Several of these antibodies have been
deposited with ATCC, as disclosed herein. In one embodiment, the
20 monoclonal antibodies of the invention will have the same biological
characteristics as the monoclonal antibodies secreted by the hybridoma
cell line(s) deposited under Accession No. HB-12456, HB-12535, HB-12534,
or HB-12536. The term "biological characteristics" is used to refer to
the *in vitro* and/or *in vivo* activities or properties of the monoclonal
25 antibody, such as the ability to specifically bind to Apo-2 or to
substantially block, induce or enhance Apo-2 activation. Particular
activities and properties of various anti-Apo-2 antibodies are described
in further detail in the Examples below. To determine whether a
monoclonal antibody has the same specificity as the 3F11.39.7 antibody
30 specifically disclosed, for instance, one can compare activity in Apo-2
blocking and apoptosis induction assays, such as those described in the
Examples below. The monoclonal antibody preferably has the
hypervariable region residues of one or more of the above-mentioned ATCC
deposited antibodies, e.g., it may comprise a humanized variant.
35 Chimeric and humanized Apo-2 antibodies derived from, constructed from,
or containing sequence, regions or domains from any of the above-
mentioned deposited antibodies are considered within the scope of the
invention. Such chimeric or humanized Apo-2 antibodies may be prepared
using techniques known in the art and further described below.

40 The antibodies of the invention may also comprise monovalent
antibodies. Methods for preparing monovalent antibodies are well known
in the art. For example, one method involves recombinant expression of

5 immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

10 *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348
15 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an F(ab')₂ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

20 The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH₁) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH₁ domain including one or more cysteines from the
25 antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical
30 couplings of antibody fragments are also known.

3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin
35 chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are
40 replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human

5 immunoglobulin are replaced by corresponding non-human residues.
Humanized antibodies may also comprise residues which are found neither
in the recipient antibody nor in the imported CDR or framework
sequences. In general, the humanized antibody will comprise
substantially all of at least one, and typically two, variable domains,
10 in which all or substantially all of the CDR regions correspond to those
of a non-human immunoglobulin and all or substantially all of the FR
regions are those of a human immunoglobulin consensus sequence. The
humanized antibody optimally also will comprise at least a portion of an
immunoglobulin constant region (Fc), typically that of a human
15 immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et
al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol.,
2:593-596 (1992)].

20 Methods for humanizing non-human antibodies are well known in
the art. Generally, a humanized antibody has one or more amino acid
residues introduced into it from a source which is non-human. These
non-human amino acid residues are often referred to as "import"
residues, which are typically taken from an "import" variable domain.
Humanization can be essentially performed following the method of Winter
and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et
25 al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-
1536 (1988)], by substituting rodent CDRs or CDR sequences for the
corresponding sequences of a human antibody. Accordingly, such
"humanized" antibodies are chimeric antibodies (U.S. Patent No.
4,816,567), wherein substantially less than an intact human variable
30 domain has been substituted by the corresponding sequence from a non-
human species. In practice, humanized antibodies are typically human
antibodies in which some CDR residues and possibly some FR residues are
substituted by residues from analogous sites in rodent antibodies.

35 The choice of human variable domains, both light and heavy,
to be used in making the humanized antibodies is very important in order
to reduce antigenicity. According to the "best-fit" method, the
sequence of the variable domain of a rodent antibody is screened against
the entire library of known human variable domain sequences. The human
sequence which is closest to that of the rodent is then accepted as the
40 human framework (FR) for the humanized antibody [Sims et al., J.
Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901
(1987)]. Another method uses a particular framework derived from the

5 consensus sequence of all human antibodies of a particular subgroup of
light or heavy chains. The same framework may be used for several
different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci.
USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

10 It is further important that antibodies be humanized with
retention of high affinity for the antigen and other favorable
biological properties. To achieve this goal, according to a preferred
method, humanized antibodies are prepared by a process of analysis of
the parental sequences and various conceptual humanized products using
15 three dimensional models of the parental and humanized sequences. Three
dimensional immunoglobulin models are commonly available and are
familiar to those skilled in the art. Computer programs are available
which illustrate and display probable three-dimensional conformational
structures of selected candidate immunoglobulin sequences. Inspection
of these displays permits analysis of the likely role of the residues in
20 the functioning of the candidate immunoglobulin sequence, i.e., the
analysis of residues that influence the ability of the candidate
immunoglobulin to bind its antigen. In this way, FR residues can be
selected and combined from the consensus and import sequence so that the
desired antibody characteristic, such as increased affinity for the
25 target antigen(s), is achieved. In general, the CDR residues are
directly and most substantially involved in influencing antigen binding
[see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon
immunization, of producing a full repertoire of human antibodies in the
30 absence of endogenous immunoglobulin production can be employed.
Transfer of the human germ-line immunoglobulin gene array in such germ-
line mutant mice will result in the production of human antibodies upon
antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci.
USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993);
35 Bruggemann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can
also be produced in phage display libraries [Hoogenboom and Winter, J.
Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581
(1991)]. The techniques of Cole et al. and Boerner et al. are also
available for the preparation of human monoclonal antibodies (Cole et
40 al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77
(1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

5

4. Bispecific Antibodies

10
20
30
40

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a

5 hybrid immunoglobulin heavy chain with a first binding specificity in
one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair
(providing a second binding specificity) in the other arm. It was found
that this asymmetric structure facilitates the separation of the desired
10 bispecific compound from unwanted immunoglobulin chain combinations, as
the presence of an immunoglobulin light chain in only one half of the
bispecific molecule provides for a facile way of separation. This
approach is disclosed in WO 94/04690 published 3 March 1994. For further
details of generating bispecific antibodies see, for example, Suresh et
al., Methods in Enzymology, 121:210 (1986).

15 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the
present invention. Heteroconjugate antibodies are composed of two
covalently joined antibodies. Such antibodies have, for example, been
proposed to target immune system cells to unwanted cells [US Patent No.
20 4,676,980], and for treatment of HIV infection [WO 91/00360; WO
92/200373; EP 03089]. It is contemplated that the antibodies may be
prepared *in vitro* using known methods in synthetic protein chemistry,
including those involving crosslinking agents. For example,
immunotoxins may be constructed using a disulfide exchange reaction or
25 by forming a thioether bond. Examples of suitable reagents for this
purpose include iminothiolate and methyl-4-mercaptobutyrimidate and
those disclosed, for example, in U.S. Pat. No. 4,676,980.

30 6. Triabodies

Triabodies are also within the scope of the invention. Such
antibodies are described for instance in Iliades et al., FEBS Letters,
409:437-441 (1997) and Korrt et al., Protein Engineering, 10:423-433
(1997).

35 7. Conjugates

The invention also pertains to immunoconjugates comprising
Apo-2 antibody described herein conjugated to a cytotoxic agent such
as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin
of bacterial, fungal, plant or animal origin, or fragments thereof),
or a radioactive isotope (i.e., a radioconjugate). Chemotherapeutic
agents useful in the generation of such immunoconjugates have been
40 described above. Enzymatically active toxins and fragments thereof
which can be used include diphtheria A chain, nonbinding active
fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas*

5 aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes.

10 A variety of radionuclides are available for the production of radioconjugate antibodies. Examples include ^{212}Bi , ^{131}I , ^{131}In , ^{90}Y and ^{186}Re .

15 Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutarelddehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science, 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

20
25

30 In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the mammal, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

35 Immunoliposomes comprising Apo-2 antibody may also be prepared. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

40 Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through

5 filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. J. Biol. Chem. 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained
10 within the liposome. See Gabizon et al. J. National Cancer Inst. 81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see
15 WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

20 Enzymes that are useful in this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the
25 anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents;
30 carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs
35 derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs [see, e.g., Massey, Nature 328: 457-458 (1987)].
40 Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

5 The enzymes of this invention can be covalently bound to the antibody by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen-binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art [see, e.g., Neuberger et al., Nature, 312: 604-608 (1984)].

10 In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life.

15 This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

20 The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment. See, e.g., U.S. Patent No. 5,747,035.

25 Covalent modifications of the antibody are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

30 The antibodies may optionally be covalently attached or conjugated to one or more chemical groups. A polyol, for example, can be

5 conjugated to an antibody molecule at one or more amino acid residues,
including lysine residues as disclosed in WO 93/00109. Optionally, the
polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG),
however, those skilled in the art recognize that other polyols, such as,
for example, poly(propylene glycol) and polyethylene-polypropylene
10 glycol copolymers, can be employed using techniques for conjugating PEG
to polypeptides. A variety of methods for pegylating polypeptides have
been described. See, e.g. U.S. Patent No. 4,179,337 which discloses the
conjugation of a number of hormones and enzymes to PEG and polypropylene
glycol to produce physiologically active compositions having reduced
15 immunogenicities.

The antibodies may also be fused or linked to another heterologous
polypeptide or amino acid sequence such as an epitope tag.

8. Other Modifications

Other modifications of the Apo-2 antibodies are contemplated.
For example, it may be desirable to modify the antibodies of the
invention with respect to effector function, so as to enhance the
therapeutic effectiveness of the antibodies. For instance, cysteine
residue(s) may be introduced into the Fc region, thereby allowing
interchain disulfide bond formation in this region. The homodimeric
antibody thus generated may have improved internalization capability
and/or increased complement-mediated cell killing [see, e.g., Caron et
al., J. Exp. Med., 176:1191-1195 (1992); Shopes, J. Immunol., 148:2918-
2922 (1992). Homodimeric antibodies may also be prepared using
heterobifunctional cross-linkers as described in Wolff et al., Cancer
Research, 53:2560-2565 (1993). Ghetie et al., Proc. Natl. Acad. Sci.,
94:7509-7514 (1997), further describe preparation of IgG-IgG homodimers
and disclose that such homodimers can enhance apoptotic activity as
compared to the monomers. Alternatively, the antibodies can be
engineered to have dual Fc regions [see, Stevenson et al., Anti-Cancer
Drug Design, 3:219-230 (1989)].

D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic
utility. Agonistic Apo-2 antibodies, for instance, may be employed to
activate or stimulate apoptosis in cancer cells.

The antibody is preferably administered to the mammal in
a carrier. Suitable carriers and their formulations are described in
Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing

5 Co., edited by Oslo et al. Typically, an appropriate amount of a
pharmaceutically-acceptable salt is used in the formulation to render
the formulation isotonic. Examples of a pharmaceutically-acceptable
carrier include saline, Ringer's solution and dextrose solution. The
10 pH of the solution is preferably from about 5 to about 8, and more
preferably from about 7 to about 7.5. Further carriers include
sustained release preparations such as semipermeable matrices of solid
hydrophobic polymers containing the antibody, which matrices are in
the form of shaped articles, e.g., films, liposomes or microparticles.
15 It will be apparent to those persons skilled in the art that certain
carriers may be more preferable depending upon, for instance, the
route of administration and concentration of antibody being
administered.

20 The antibody can be administered to the mammal by injection
(e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or
by other methods such as infusion that ensure its delivery to the
bloodstream in an effective form. The antibody may also be
administered by intratumoral, peritumoral, intralesional, or
perilesional routes, to exert local as well as systemic therapeutic
effects. Local or intravenous injection is preferred.

25 Effective dosages and schedules for administering the
antibody may be determined empirically, and making such determinations
is within the skill in the art. Those skilled in the art will
understand that the dosage of antibody that must be administered will
vary depending on, for example, the mammal which will receive the
30 antibody, the route of administration, the particular type of antibody
used and other drugs being administered to the mammal. Guidance in
selecting appropriate doses for antibody is found in the literature on
therapeutic uses of antibodies, e.g., Handbook of Monoclonal
Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge,
35 N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human
Diagnosis and Therapy, Haber et al., eds., Raven Press, New York
(1977) pp. 365-389. A typical daily dosage of the antibody used alone
might range from about 1 µg/kg to up to 100 mg/kg of body weight or
more per day, depending on the factors mentioned above.

40 The antibody may also be administered to the mammal in
combination with effective amounts of one or more other therapeutic

5 agents or in conjunction with radiation treatment. Therapeutic agents
contemplated include chemotherapeutics as well as immunoadjuvants and
cytokines. The antibody may be administered sequentially or
concurrently with the one or more other therapeutic agents. The
10 amounts of antibody and therapeutic agent depend, for example, on what
type of drugs are used, the cancer being treated, and the scheduling
and routes of administration but would generally be less than if each
were used individually.

15 Following administration of antibody to the mammal, the
mammal's cancer and physiological condition can be monitored in
various ways well known to the skilled practitioner. For instance,
tumor mass may be observed physically or by standard x-ray imaging
techniques.

20 The Apo-2 receptor antibodies of the invention may also be
useful in enhancing immune-mediated cell death in cells expressing Apo-2
receptor(s), for instance, through complement fixation or ADCC.
Alternatively, antagonistic anti-Apo-2 receptor antibodies may be used
to block excessive apoptosis (for instance in neurodegenerative disease)
or to block potential autoimmune/inflammatory effects of Apo-2 resulting
from NF- κ B activation. Such therapeutic antibodies can be utilized
25 according to the methods and techniques described above.

30 Apo-2 antibodies may further be used in diagnostic assays for
Apo-2, e.g., detecting its expression in specific cells, tissues, or
serum. Various diagnostic assay techniques known in the art may be
used, such as competitive binding assays, direct or indirect sandwich
assays and immunoprecipitation assays conducted in either heterogeneous
or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of
Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in
the diagnostic assays can be labeled with a detectable moiety. The
detectable moiety should be capable of producing, either directly or
35 indirectly, a detectable signal. For example, the detectable moiety may
be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or
chemiluminescent compound, such as fluorescein isothiocyanate,
rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase,
beta-galactosidase or horseradish peroxidase. Any method known in the
40 art for conjugating the antibody to the detectable moiety may be
employed, including those methods described by Hunter et al., Nature,

5 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

10 Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

15 E. Kits Containing Apo-2 or Apo-2 Antibodies

20 In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

35 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

40 The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

 All patent and literature references cited in the present

5 specification are hereby incorporated by reference in their entirety.

EXAMPLES

10 All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

EXAMPLE 1

Isolation of cDNA clones Encoding Human Apo-2

20 Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows. Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xho1, Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

35 The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as per the manufacturer's recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the

5 colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

10 An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then removed, the pellet dried in a speedvac and resuspended in distilled water (10 ml).

20 The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

30 The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was

5 removed and the residue pellet was dried in a speedvac and resuspended
in distilled water (3 ml). The ligated cDNA/pSST-amy.1 vector DNA was
chilled on ice to which was added electrocompetent DH10B bacteria (Life
Tech., 20 ml). The bacteria vector mixture was then electroporated as
recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1
10 ml) was added and the mixture was incubated at 37°C for 30 minutes. The
transformants were then plated onto 20 standard 150 mm LB plates
containing ampicillin and incubated for 16 hours (37°C). Positive
colonies were scraped off the plates and the DNA was isolated from the
bacterial pellet using standard protocols, e.g. CsCl-gradient.

15 The cDNA libraries were screened by hybridization with a
synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGCGGG
(SEQ ID NO:3) based on the EST.

20 Three cDNA clones were sequenced in entirety. The
overlapping coding regions of the cDNAs were identical except for codon
410 (using the numbering system for Fig. 1); this position encoded a
leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue
(ATG) in the kidney cDNA, possibly due to polymorphism.

25 The entire nucleotide sequence of Apo-2 is shown in Figure 1
(SEQ ID NO:2). Clone 27868 (also referred to as PRK5-Apo-2 deposited as
ATCC 209021, as indicated below) contains a single open reading frame
with an apparent translational initiation site at nucleotide positions
140-142 [Kozak et al., supra] and ending at the stop codon found at
nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted
30 polypeptide precursor is 411 amino acids long, a type I transmembrane
protein, and has a calculated molecular weight of approximately 45 kDa.
Hydropathy analysis (not shown) suggested the presence of a signal
sequence (residues 1-53), followed by an extracellular domain (residues
54-182), a transmembrane domain (residues 183-208), and an intracellular
35 domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid
sequence analysis of Apo-2-IgG expressed in 293 cells showed that the
mature polypeptide starts at amino acid residue 54, indicating that the
actual signal sequence comprises residues 1-53. Apo-2 polypeptide is
obtained or obtainable by expressing the molecule encoded by the cDNA
40 insert of the deposited ATCC 209021 vector.

TNF receptor family proteins are typically characterized by

5 the presence of multiple (usually four) cysteine-rich domains in their
extracellular regions -- each cysteine-rich domain being approximately
45 amino acids long and containing approximately 6, regularly spaced,
cysteine residues. Based on the crystal structure of the type 1 TNF
10 receptor, the cysteines in each domain typically form three disulfide
bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are
paired together. Like DR4, Apo-2 contains two extracellular cysteine-
rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR
family members contain three or more such domains [Smith et al., Cell,
76:959 (1994)].

15 The cytoplasmic region of Apo-2 contains a death domain
(amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which
shows significantly more amino acid sequence identity to the death
domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%);
or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids
20 that are required for signaling by TNFR1 [Tartaglia et al., supra] are
conserved in Apo-2 while the other two residues are semi-conserved (see
Fig. 2B).

25 Based on an alignment analysis (using the ALIGNTM computer
program) of the full-length sequence, Apo-2 shows more sequence identity
to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1
(19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP)
(29%).

EXAMPLE 2

A. Expression of Apo-2 ECD

30 A soluble extracellular domain (ECD) fusion construct was
prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1)
was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma).
(The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1
35 to provide flexibility at the junction, even though residues 183 and 184
are predicted to be in the transmembrane region). The Flag epitope-
tagged molecule was then inserted into pRK5, and expressed by transient
transfection into human 293 cells (ATCC CRL 1573).

40 After a 48 hour incubation, the cell supernatants were
collected and either used directly for co-precipitation studies (see
Example 3) or subjected to purification of the Apo-2 ECD-Flag by
affinity chromatography on anti-Flag agarose beads, according to

5 manufacturer's instructions (Sigma).

B. Expression of Apo-2 ECD as an Immunoadhesin

10 A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G₁ heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al.,
15 supra.

EXAMPLE 3

Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

20 To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then
25 analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By
30 using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then
35 detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

40 The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIAcore™

5 instrument. The BIAcore™ analysis indicated a dissociation constant
(K_d) of about 1 nM. BIAcore™ analysis also showed that the Apo-2 ECD is
not capable of binding other apoptosis-inducing TNF family members,
namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712
(1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis
10 Biochemicals). The data thus shows that Apo-2 is a specific receptor
for Apo-2L.

EXAMPLE 4

Induction of Apoptosis by Apo-2

15 Because death domains can function as oligomerization
interfaces, over-expression of receptors that contain death domains may
lead to activation of signaling in the absence of ligand [Frazer et al.,
supra, Nagata et al., supra]. To determine whether Apo-2 was capable of
inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were
20 transiently transfected by calcium phosphate precipitation (293 cells)
or electroporation (HeLa cells) with a pRK5 vector or pRK5-based
plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount
of plasmid DNA was adjusted by adding vector DNA. Apoptosis was
assessed 24 hours after transfection by morphology (Fig. 4A); DNA
25 fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine
exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669
(1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells
underwent marked apoptosis.

30 For samples assayed by FACS, the HeLa cells were co-
transfected with pRK5-CD4 as a marker for transfection and apoptosis was
determined in CD4-expressing cells; FADD was co-transfected with the
Apo-2 plasmid; the data are means ± SEM of at least three experiments,
as described in Marsters et al., Curr. Biol., 6:1669 (1996). The
caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research
35 Biochemicals Intl.) were added at 200 µM at the time of transfection.
As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-
fmk blocked apoptosis induction by Apo-2, indicating the involvement of
Ced-3-like proteases in this response.

40 FADD is an adaptor protein that mediates apoptosis
activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but
does not appear necessary for apoptosis induction by Apo-2L [Marsters et

5 al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant
form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-
3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al.,
supra] did not inhibit apoptosis induction by Apo-2 when co-transfected
10 into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2
signals apoptosis independently of FADD. Consistent with this
conclusion, a glutathione-S-transferase fusion protein containing the
Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and
translated FADD (data not shown).

15 EXAMPLE 5

Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 µg/ml, prepared as described in Pitti et
al., supra) was pre-incubated for 1 hour at room temperature with PBS
20 buffer or affinity-purified Apo-2 ECD (5 µg/ml) together with anti-Flag
antibody (Sigma) (1 µg/ml) and added to HeLa cells. After a 5 hour
incubation, the cells were analyzed for apoptosis by FACS (as above)
(Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the
soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D),
25 confirming a specific interaction between Apo-2L and Apo-2. Similar
results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-
response analysis showed half-maximal inhibition at approximately 0.3 nM
Apo-2 immunoadhesin (Fig. 4E).

30 EXAMPLE 6

Activation of NF-KB by Apo-2

An assay was conducted to determine whether Apo-2 activates
NF-KB.

HeLa cells were transfected with pRK5 expression plasmids
35 encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested
24 hours after transfection. Nuclear extracts were prepared and 1 µg of
nuclear protein was reacted with a ³²P-labelled NF-KB-specific synthetic
oligonucleotide probe

ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al.,
40 J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold

5 excess of unlabelled probe, or with an irrelevant ³²P-labelled synthetic oligonucleotide
AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples,
antibody to p65/RelA subunits of NF-KB (1 µg/ml; Santa Cruz
Biotechnology) was added. DNA binding was analyzed by an
10 electrophoretic mobility shift assay as described by Hsu et al., supra;
Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon
transfection into HeLa cells, both Apo-2 and DR4 induced significant NF-
KB activation as measured by the electrophoretic mobility shift assay;
15 the level of activation was comparable to activation observed for Apo-
3/DR3. Antibody to the p65/RelA subunit of NF-KB inhibited the mobility
of the NF-KB probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can
regulate NF-KB activity. HeLa cells or MCF7 cells (human breast
20 adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer,
soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see
Pennica et al., Nature, 312:721 (1984)) (1 µg/ml) and assayed for NF-KB
activity as above. The results are shown in Fig. 5B. The Apo-2L
induced a significant NF-KB activation in the treated HeLa cells but not
25 in the treated MCF7 cells; the TNF-alpha induced a more pronounced
activation in both cell lines. Several studies have disclosed that NF-
KB activation by TNF can protect cells against TNF-induced apoptosis
[Nagata, supra].

The effects of a NF-KB inhibitor, ALLN (N-acetyl-Leu-Leu-
30 norleucinal) and a transcription inhibitor, cyclohexamide, were also
tested. The HeLa cells (plated in 6-well dishes) were preincubated with
PBS buffer, ALLN (Calbiochem) (40 µg/ml) or cyclohexamide (Sigma) (50
µg/ml) for 1 hour before addition of Apo-2L (1 µg/ml). After a 5 hour
incubation, apoptosis was analyzed by FACS (see Fig. 5C).

35 The results are shown in Fig. 5C. Both ALLN and
cyclohexamide increased the level of Apo-2L-induced apoptosis in the
HeLa cells. The data indicates that Apo-2L can induce protective NF-KB-
dependent genes. The data also indicates that Apo-2L is capable of

5 activating NF-KB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

EXAMPLE 7

Northern Blot Analysis

10 Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase ³²P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II
15 (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute
20 wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was
25 detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been
30 shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

EXAMPLE 8

Chromosomal Localization of the Apo-2 gene

35 Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al.,
40 Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to

5 the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to
D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4
showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00),
which maps also to human chromosome 8p21.

EXAMPLE 9

Preparation of Monoclonal Antibodies Specific for Apo-2

Balb/c mice (obtained from Charles River Laboratories) were
immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein
(diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research
Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day
intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing
an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in
Fig. 1) to the hinge and Fc region of human immunoglobulin G₁ heavy
chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl.
Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin protein was
expressed by transient transfection into human 293 cells and purified
from cell supernatants by protein A affinity chromatography, as
described by Ashkenazi et al., supra (See also Example 2B above).

Three days after the final boost, popliteal lymph nodes were
removed from the mice and a single cell suspension was prepared in DMEM
media (obtained from Biowhitakker Corp.) supplemented with 1%
penicillin-streptomycin. The lymph node cells were then fused with
murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene
glycol and cultured in 96-well culture plates. Hybridomas resulting
from the fusion were selected in HAT medium. Ten days after the fusion,
hybridoma culture supernatants were screened in an ELISA to test for the
presence of monoclonal antibodies binding to the Apo-2 ECD immunoadhesin
protein.

In the ELISA, 96-well microtiter plates (Maxisorb; Nunc,
Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-
human IgG Fc (purchased from Cappel Laboratories) in PBS to each well
and incubating at 4°C overnight. The plates were then washed three
times with wash buffer (PBS containing 0.05% Tween 20). The wells in
the microtiter plates were then blocked with 200 µl of 2.0% bovine serum
albumin in PBS and incubated at room temperature for 1 hour. The plates

5 were then washed again three times with wash buffer.

After the washing step, 50 μ l of 0.4 μ g/ml Apo-2 ECD immunoadhesin protein (as described above) in assay buffer was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with wash buffer.

10 Following the wash steps, 100 μ l of the hybridoma supernatants or various concentrations of purified antibody (using Protein A-sepharose columns) was added to designated wells in the presence of CD4-IgG. 100 μ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were
15 incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50 μ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50 μ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped
20 by adding 50 μ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

The supernatants testing positive in the ELISA (calculated as approximately 4 times above background) were further analyzed by FACS
30 analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25 μ l of cells suspended (at 4×10^6 cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02% NaN_3) were added to U-bottom microtiter wells, mixed with 100 μ l of culture supernatant or purified
35 antibody (purified on Protein A-sepharose columns) (10 μ g /ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100 μ l FITC-conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150 μ l of cell sorter buffer and then analyzed by FACScan (Becton Dickinson,

5 Mountain View, CA).

Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.

EXAMPLE 10

Assay for Ability of Apo-2 Abs to Agonistically induce Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis. The 9D cells (5×10^5 cells/0.1ml) were incubated with varying concentrations of antibodies in 100 μ l complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C with or without 10 μ g of goat anti-mouse IgG Fc antibody (Cappel Laboratories) in 300 μ l of complete RPMI. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO₂. The cells were then harvested and washed once with PBS. The apoptosis of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200 μ l binding buffer. Ten μ l of annexin-V-FITC (1 μ g/ml) and 10 μ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).

Assay for Antibody Ability to Block Apo-2 ligand-induced Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis. The 9D cells (5×10^5 cells/0.1ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, penicillin, streptomycin, sodium pyruvate) and placed into individual Falcon 2052 tubes. Cells were preincubated with 10 μ g of antibodies in 200 μ l media for 15 minutes on ice. 0.2 ml of Apo-2 ligand (2.5 μ g/ml) (soluble His-tagged Apo-2L prepared as described in WO 97/25428; see also Pitti et al., supra) was suspended into complete RPMI media, and then added into the tubes containing the 9D cells. The 9D cells were incubated overnight at 37°C and in the presence of 7% CO₂. The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech).

Specifically, the cells were washed in PBS and resuspended in 200 μ l binding buffer. Ten μ l of annexin-V-FITC (1 μ g/ml) and 10 μ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

The results are shown in Figure 10. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either Apo-2 or the DR4 receptor. Thus, to detect any blocking activity of the Apo-2 antibodies, the interaction between DR4 and Apo-2L needed to be blocked. In combination with the anti-DR4 antibody, 4H6.17.8 (ATCC HB-12455), the Apo-2 antibody 3F11.39.7 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activities of these two antibodies by themselves, as shown in Figure 10. Accordingly, it is believed that the 3F11.39.7 antibody is a blocking Apo-2 antibody.

5

EXAMPLE 12

ELISA Assay to Test Binding of Apo-2 Antibodies to Other Apo-2 Ligand Receptors

10 An ELISA was conducted to determine if the monoclonal antibody described in Example 9 was able to bind other known Apo-2L receptors beside Apo-2. Specifically, the 3F11.39.7 antibody was tested for binding to DR4 [Pan et al., supra], DcR1 [Sheridan et al., supra], and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006 (1997)]. The ELISA was performed essentially as described in Example 9 above.

15 The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.

EXAMPLE 13

Antibody Isotyping

20 The isotype of the 3F11.39.7 antibody (as described above) was determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C. The plates were then washed with wash buffer (as described in Example 9 above). The wells in the microtiter plates were then blocked with 200
25 μ l of 2% bovine serum albumin and incubated at room temperature for one hour. The plates were washed again three times with wash buffer. Next, 100 μ l of 5 μ g/ml of purified 3F11.39.7 antibody was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 μ l HRP-conjugated goat anti-mouse IgG (as described above) was
30 added to each well. The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

The isotyping analysis showed that the 3F11.39.7 antibody is an IgG1 antibody.

35

EXAMPLE 14

Preparation of Monoclonal Antibodies Specific for DR4

40 Further monoclonal antibodies to Apo-2 were produced essentially as described in Example 9 above. Using the capture ELISA described in Example 9, additional anti-Apo-2 antibodies, referred to as 2B3.7.1; 4B9.23.6; 5C7.9.1; 3C9.8.6; and 4H10.14.10, were identified (see Table

5 in Figure 12). Analysis by FACS (using the technique described in
Example 9) confirmed binding of these antibodies to 9D cells
expressing Apo-2 (data not shown).

10 Three additional anti-Apo-2 antibodies, referred to as 3H1.18.10,
3H3.14.5 and 3D5.1.10, were produced using a mixed antigen
immunization protocol as follows. Animals were immunized with four
receptor immunoadhesins - DR4 [Pan et al., Science, 276:111-113
(1997)]; Apo-2 [as described herein]; DcR1 [Sheridan et al., Science
277:818-821 (1997)]; and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006
(1997)]. Receptor immunoadhesins (designated "DR4-IgG", "Apo-2-IgG",
15 "DcR1-IgG" and "DcR2-IgG") were prepared by fusing the extracellular
domain sequence of each receptor to the hinge and Fc region of human
immunoglobulin G₁ heavy chain in pRK5 as described previously [Ashkenazi
et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The
immunoadhesin proteins were expressed by transient transfection into
20 human 293 cells and purified from cell supernatants by protein A
affinity chromatography, as described by Ashkenazi et al., *supra*.
Purified immunoadhesin was suspended in phosphate buffered saline (PBS).

25 Balb/C mice (from Charles River Laboratories) were immunized into
each hind foot pad 14 times at 3-4 day intervals, with a mixture of
DR4-IgG, Apo-2-IgG, DcR1-IgG and DcR2-IgG (1 µg each) suspended in
monophosphoryl lipid A plus trehalose dicorynomycolate adjuvant (MPL-
TDM; Ribi Immunochem. Research Inc., Hamilton, MT) at a 1:1 ratio of
immunoadhesin:adjuvant (DcR2-IgG was only included in the mixture
used for the final six immunizations).

30 Three days after the final boost, popliteal lymph node cells
nodes were removed from the mice and a single cell suspension was
prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented
with 1% penicillin-streptomycin. The lymph node cells were fused with
murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene
35 glycol and cultured in 96-well culture plates.

Hybridomas were selected in super DMEM [DMEM plus 10% fetal calf
serum (FCS), 10% NCTC-109 (BioWittaker, Wakersville, MD), 100 mM
pyruvate, 100 U/ml insulin, 100 mM oxaloacetic acid, 2 mM glutamine,
1% nonessential amino acids (GIBCO), 100 U/ml penicillin and 100 µg/ml
40 streptomycin] containing 100 µM hypoxanthine, 0.4 µM aminopterin, and

5 16 μ M thymidine (1x HAT, Sigma Chemical Co., St. Louis, MO).

Ten days after the fusion, 180 μ l of each hybridoma culture supernatant was screened for the presence of antibodies to three different antigens (*i.e.* DR4-IgG, Apo-2-IgG and CD4-IgG control) in a capture ELISA. Hybridoma cells were re-fed with 200 μ l of super DMEM containing 10% FCS and antibiotics. Two days later, 180 μ l of culture supernatant was collected and screened for the presence of antibodies to another two different antigens (*i.e.* DcR1-IgG and DcR2-IgG) in a capture ELISA. After careful examination of the ELISA results, potential positive hybridomas secreting monoclonal antibodies against each antigen were cloned twice using a limiting dilution method.

EXAMPLE 15

Antibody Isotyping

The isotypes of the 2B3.7.1; 4B9.23.6; 5C7.9.1; 3H1.18.10; 3C9.8.6; 4H10.14.10; 3H3.14.5 and 3D5.1.10 anti-Apo-2 antibodies (described in Example 14) were determined essentially as described in Example 13.

The isotyping analysis showed that the 2B3.7.1; 5C7.9.1; 3H1.18.10; 3H3.14.5 and 3D5.1.10 antibodies are IgG1 antibodies. Anti-Apo-2 antibodies 4B9.23.6 and 4H10.14.10 are IgG2a antibodies, and antibody 3C9.8.6 is an IgG2b antibody. (See, also, Figure 12)

EXAMPLE 16

Determination of Monoclonal Antibody Affinities

The equilibrium dissociation and association constant rates of two of the Apo-2 antibodies (described in the Examples above) were determined using KinExA™, an automated immunoassay system (Sapidyne Instruments, Inc., Boise, ID), as described with a modification by Blake et al., Journal of Biological Chemistry, 271:27677-685 (1996); and Craig et al., Journal of Molecular Biology, 281:183-201 (1998). Briefly, 1.0 ml of anti-human IgG agarose beads (56 μ m, Sigma, St. Louis, MO) were coated with 20 μ g of Apo-2-IgG (described in Example 9) in PBS by gentle mixing at room temperature for 1 hour. After washing with PBS, non-specific binding sites were blocked by incubating with 10% human serum in PBS for 1 hour at room temperature.

5 A bead pack (~4 mm high) was created in the observation flow cell
by the KinExA™ instrument. The blocked beads were diluted into 30 ml
of assay buffer (0.01% BSA/PBS). The diluted beads (550 µl) were next
drawn through the flow cell with a 20 µm screen and washed with 1 ml
of running buffer (0.01% BSA; 0.05% Tween 20 in PBS). The beads were
10 then disrupted gently with a brief backflush of running buffer,
followed by a 20 second setting period to create a uniform and
reproducible bead pack. For equilibrium measurements, the selected
Apo-2 antibodies (3F11.39.7 and 3H3.14.5) (5 ng/ml in 0.01 % BSA/PBS)
were mixed with a serial dilution of Apo-2-IgG (starting from 2.5 nM
15 to 5.0 pM) and were incubated at room temperature for 2 hours. Once
equilibrium was reached, 4.5 ml of this mixture was drawn through the
beads, followed by 250 µl of running buffer to wash out the unbound
antibodies. The primary antibodies bound to beads were detected by
1.5 ml of phycoerythrin labeled goat anti-mouse IgG (Jackson
20 ImmunoResearch). Unbound labeled material was removed by drawing 4.5
ml of 0.5 M NaCl through the bead pack over a 3 minute period. The
equilibrium constant was calculated using the software provided by the
manufacturer (Sapidyne, Inc.).

The affinity determinations for the Apo-2 antibodies are shown in
25 Figure 13. Affinity determinations for immunoadhesin constructs of
the DR4 and Apo-2 receptors for Apo-2L are shown for comparison. The
affinities (Kd-1) of the antibodies 3F11.39.7 and 3H3.14.5 were 20 pM
and 5 pM, respectively, demonstrating that both antibodies have high
affinities.

30 EXAMPLE 17

Apoptosis Assay of Human Tumor Cell Lines

The apoptotic activities of the Apo-2 monoclonal antibodies were
further examined in assays to determine the cell viability of cancer
35 cells after treatment with the antibodies or Apo-2L.

SKMES-1 cells (human lung tumor cell line; ATCC), Colo205 cells
(human colon tumor cell line; ATCC), or G142 human glioma cells (Dr.
Manfred Westphal, Universitats Krankenhaus Eppendorf, Hamburg,
Germany) were seeded at 4×10^4 cells/well in complete high glucose 50:50
40 medium supplemented with glutamine, penicillin and streptomycin, in
tissue culture plates and allowed to attach overnight at 37°C. The

5 media was then removed from the wells, and 0.1 ml of antibody (anti-Apo-2 antibodies diluted 0.001-10 microgram/ml in complete medium) was added to selected wells. Control wells without antibody received a media change with or without Apo-2L. The plates were then incubated for 1 hour at room temperature.

10 The culture supernatant was removed from the wells containing the test antibodies, and 10 microgram/ml goat anti-mouse IgG-Fc (Cappel Laboratories) was added to the wells. Media was changed in the control wells. The plates were incubated overnight at 37°C. As a control, Apo-2L (as described in Example 11) (in potassium phosphate buffer, pH 7.0) was diluted to 2 microgram/ml. 0.1 ml of the diluted Apo-2L solution was added to selected wells, and then serial three-fold dilutions were carried down the plate.

15 Culture supernatants were then removed from the wells by aspiration, and the plates were flooded with 0.5% crystal violet in methanol solution. After 15 minutes, the crystal violet solution was removed by flooding the plates with running tap water. The plates were then allowed to dry overnight.

20 Absorbance was read on an SLT 340 ATC plate reader (Salzburg, Austria) at 540 nm. The data was analyzed using an Excel macro and 4p-fit. The results illustrating the activity of the Apo-2 antibodies on SKMES cells are shown in Figure 14. Figure 14 shows that all the monoclonal antibodies tested (except 3H1.18.10) induced apoptosis when the monoclonal antibodies were linked by the addition of goat anti-mouse IgG-Fc antibodies. The most potent apoptotic activity was observed with antibody 3H3.14.5. The EC₅₀ of the 3H3.14.5 antibody was approximately 10 ng/ml, while the EC₅₀ of Apo-2 ligand was approximately 100 ng/ml, suggesting that the 3H3.14.5 antibody may have more potent apoptotic activity than Apo-2 ligand. In the absence of the goat anti-mouse IgG-Fc linker, the monoclonal antibodies did not demonstrate significant apoptotic activity. This result suggests it may be important for oligomerization of Apo-2 receptors to occur in order to induce apoptosis.

35 The results illustrated in Figure 15A and 15B show the activity of the Apo-2 antibodies on the Colo205 colon cancer cells and glioma cells, respectively. As shown above for the SKMES cells, the antibodies 3F11.39.7 and 3H3.14.5 demonstrated potent apoptotic activities on the Colo205 cells. The 3H3.14.5 antibody appeared to be

5 more potent than Apo-2 ligand. Even more striking effects of the
3H3.14.5 and 3F11.39.7 antibodies were obtained with the glioma G142
cells (which express Apo-2 receptor but not DR4 receptor). At 1
microgram/ml, Apo-2 ligand induced approximately 50% killing of the
10 G142 cells. In contrast, the 3H3.14.5 and 3F11.39.7 antibodies (at
0.1 microgram/ml) could induce approximately 90% killing of the G142
tumor cells. These results suggest that in cancer cells expressing
Apo-2 receptor but not DR4, such anti-Apo-2 antibodies may be more
potent apoptotic inducing agents.

15 EXAMPLE 18
ELISA Assay to Test Binding of Apo-2 Antibodies to Other Apo-2L
Receptors

20 An ELISA assay was conducted (as described in Examples 9 and 12)
to determine binding of the Apo-2 antibodies to other known Apo-2L
receptors, beside Apo-2.

25 The results are shown in Figure 12 and 16. Antibody 3D5.1.10
demonstrated a significant level of binding to DR4 although it did not
bind to DcR1 and DcR2. Antibodies 3F11.39.7 and 3H1.18.10 bind Apo-2
specifically while antibody 3H3.14.5 demonstrated a low level of cross
reactivities to DR4 and DcR1.

30 EXAMPLE 19
Determination of Blocking Activity of anti-Apo-2 Antibodies

35 In a capture ELISA, 96-well microtiter plates (Maxisorb;
Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat
anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each
well and incubating at 4°C overnight. The plates were then washed three
times with wash buffer (PBS containing 0.05% Tween 20). The wells in
the microtiter plates were then blocked with 200 µl of 2.0% bovine serum
albumin in PBS and incubated at room temperature for 1 hour. The plates
were then washed again three times with wash buffer.

40 After the washing step, 100 µl of various concentrations of
anti-Apo-2 antibodies in assay buffer was added to each well. The
plates were incubated for 1 hour at room temperature on a shaker
apparatus, followed by washing three times with wash buffer.

Following the wash steps, the plate wells were incubated with

5 50 µl/well of 10 ng/ml of Apo-2L (amino acids 114-281; prepared as described in Example 11). The plates were incubated at room temperature for 1 hour on a shaker apparatus. The Apo-2L bound to the wells was then detected by the addition of biotinylated anti-Apo-2L monoclonal antibody, 2E11 (ATCC HB-12256), followed by the addition of streptavidin.

10 The results are illustrated in Figure 17. Antibodies 3H3.14.5, 3F11.39.7, 3C9.8.6, and 4H10.14.10 inhibited the binding of Apo-2 ligand to the Apo-2 receptor captured to the goat anti human IgG-coated ELISA wells, demonstrating that these antibodies are blocking antibodies. Antibody 3H3.14.5 appeared to have the strongest blocking activity. The blocking activities of the antibodies are also summarized in Figure 12.

EXAMPLE 20

In vivo Activity of Apo-2 Antibodies

20 Colo205 cells (human colon tumor cell line; ATCC) were grown in high glucose F-12:DMEM (50:50) medium supplemented with 10% FCS, 2 mM glutamine, 100 µg/ml of penicillin, and 100 µg/ml streptomycin. The cells were harvested after treating with cell dissociation medium (Sigma, IAC) for 5 minutes. After washing in PBS, the tumor cells were resuspended in PBS at a concentration of 3×10^7 cells/ml.

25 Nude mice were injected with $3-5 \times 10^6$ cells subcutaneously in the dorsal area in a volume of 0.1 ml. Control animals received either a control IgG1 antibody or PBS vehicle alone. When the tumor size in the Colo205 tumor bearing animals became a desired size, the mice were injected i.p. with 100 µg of 3F11.39.7 or 3H3.14.5 anti-Apo-2 antibody in PBS three times per week, and the tumor sizes were measured three times/week.

30 The results illustrated in Figure 18 show that both 3F11.39.7 antibody and 3H3.14.5 antibody inhibited growth of Colo205 tumors. Compared to the tumor growth in the group of mice treated with control IgG1 or none, the group of mice treated with antibody 3H3.14.5 or 3F11.39.7 showed almost no detectable tumor growth. On Day 20, four out of eight mice showed no visible tumor growth after treatment with antibody 3H3.14.5, while two out of eight mice showed no visible tumor growth after treatment with antibody 3F11.39.7. The results suggest

5 that the monoclonal antibodies alone may be potent anti-cancer
therapeutic agents. It is presently believed that component(s) in the
treated animals' serum may link the administered (IgG1) monoclonal
antibodies, resulting in the potent agonistic anti-cancer activity
observed.

EXAMPLE 21

Crystal Structure Analysis of Apo-2 Receptor/Apo-2L Complex

Experiments were conducted to determine the crystal structure of
the complex between Apo-2L and an extracellular domain sequence of
Apo-2. As described below, the structure revealed three thin,
elongated receptors snuggled into long crevices between pairs of
monomers of the homotrimeric Apo-2 ligand. The interface is divided
into two distinct patches, one near the "bottom" of the complex close
to the receptor cell surface and one near the "top" closest to the
ligand cell surface. Both patches contain residues that are believed
to be critical for high-affinity binding. It is believed that the top
patch may recognize conserved hydrophobic features, while the bottom
patch may control specificity and cross-reactivity.

1. Expression and purification

Apo-2L (residues 114-281; amino acid sequence provided in Pitti
et al., supra) was expressed in *E. coli* and purified as described in
Ashkenazi et al., J. Clin. Invest., 104:155-162 (1999). Apo-2
(residues 1-130; see Fig. 1; SEQ ID NO:1) was expressed in Hi5 insect
cells (Expression Systems LLC, Woddlan, CA) with a baculovirus
transfer vector (PharMingen, San Diego, CA) under the control of a
polyhedron promoter. Protein was secreted from the cells grown at
27°C over 72 hours. The Apo-2-containing medium was separated from
the cells by centrifugation. The supernatant was run over a Q-
sepharose (Pharmacia) column and the protein eluted with a 0-1M NaCl
gradient in 20mM Tris-HCl, pH 8.0. The fractions containing Apo-2
were pooled and loaded onto a CNBr-Apo-2L affinity column. The column
was washed with 0.5M NaCl in 20mM Tris-HCL, pH 8.0, and Apo-2 was
eluted with 2M KSCN in 50mM Tris, pH 8.0. Apo-2 was further purified
by size exclusion chromatography (S-200, Pharmacia). Apo-2L in 20mM
Tris-HCl, pH 8.0 was added to purified Apo-2 in approximately
equimolar concentrations, and the complex was purified by size

5 exclusion chromatography S-75, Pharmacia) in 100 mM NaCl, 20mM Tris-
HCl, pH 8.0. The fraction containing the Apo-2L/Apo-2 complex was
further purified by anion exchange chromatography (MonoQ, Pharmacia)
and eluted with a 0-1M NaCl gradient in 20mM Tris, pH 8.0. The
complex was then concentrated to approximately 3.7 mg/mL and buffered
10 with 20mM Tris-HCl, pH 8.0, 0.1 M NaCl.

2. Crystallization and data collection

Crystals of the Apo-2L/Apo-2 complex were grown by vapor
diffusion at 19°C using the hanging drop method. The initial crystals
were grown in condition 37 of the Hampton Crystal Screen II (10% PEG
15 8000, 8% ethylene glycol, 0.1 M Hepes, pH 7.5). The crystals used for
data collection were grown by mixing 2 microliter of protein solution
with 2 microliter of reservoir consisting of 15% PEG 8000, 10%
ethylene glycol, 0.2M ammonium sulfate, 0.1M Tris-HCl, pH 7.5, and
grew to a size of 0.3 mm x 0.15 mm x 0.1 mm. The crystals were
20 transferred briefly to a droplet containing reservoir solution with
20% glycerol before flash-cooling in liquid nitrogen. The crystals
belonged to space group P2₁2₁2₁ and had unit cell dimensions a=66.8 Å,
b= 112.0 Å, c=130.8 Å. The asymmetric unit contained one Apo-2L
trimer and three receptor molecules. A 3.5 Å data set was collected
25 on a MAR imaging plate system using a Rigaku rotating anode generator
with CuKalpha radiation. A subsequent data set to 2.4 Å resolution
was collected from a single crystal at beam line 7-1 of the Stanford
Synchrotron Radiation Laboratory. The data sets were processed using
the programs in the HKL package (Otwinowski and Minor, Methods
30 Enzymol., 176:307-326 (1997)).

3. Structure determination and refinement

The Apo-2/Apo-2L structure was determined by molecular
replacement using the structure of Apo-2L alone as the search model in
combination with three-fold non-crystallographic symmetry (NCS) map
35 averaging. Using all data from 8-4 Å, the program AMoRe (CCP4, Acta
Cryst., D50:760-763 (1994)) gave a clear rotation solution with a
correlation coefficient of 30.4% (the nearest incorrect peak had a
correlation coefficient of 7.3%). The top solution to the translation
function had an initial R_{free} (Brünger, Nature, 355:472-475 (1992)) of
40 42.4% following rigid-body fitting using all data between 8 and 3.5Å.
Similar searches using the structure of Apo-2L bound to a homology

5 model of Apo-2 based on the TNF-beta-TNFR1 complex resulted in worse
molecular replacement statistics, but a three-fold NCS-averaged and
solvent-flattened map using program DM (CCP4, supra) revealed partial
density for the receptor. Three cycles of model building, NCS mask
refinement, and density averaging allowed building of receptor
10 residues 22-130. This model was refined with X-PLOR 98.1 (Brünger, X-
PLOR Manual, Version 3.1, New Haven, Connecticut, Yale University
(1992)) as modified by Molecular Simulations, Inc., using a maximum
likelihood target function, NCS constraints, positional refinement,
simulated annealing and grouped B-factors until R_{free} reached 33.5%.
15 This partially refined model was then further refined against the 2.4
Å data set, using programs X-PLOR and REFMAC (CCP4, supra).
Examination of sigma weighted 2Fo-Fc and Fo-Fc maps revealed that
differences existed among the three receptor copies, especially
between residues 100 to 130. In subsequent refinement the Apo-2L
20 trimer was subject to tight NCS restraints, while weak restraints were
applied to the most similar regions of the receptors. An overall
anisotropic B-factor correction was applied to the data as was a real
space bulk solvent correction (Brünger, X-PLOR Manual, supra). The
final model consists of three Apo-2L monomers (A, B, and D),
25 containing residues 119-131 and 144-281 (using the amino acid sequence
numbering provided in Pitti et al., supra), and Apo-2 receptor chains
R (residues 21-128) and S and T (residues 22-130). Refinement and
model statistics are shown in Figure 19. The programs Molscript and
Raster3D were used to make figures (Kraulis, J. Appl. Cryst., 24:946-
30 950 (1991); Merrit and Murphy, Acta Cryst., D50:869-873 (1994)).

The complex formed between the extracellular domain sequence of
Apo-2 (residues 1-130) and Apo-2L (residues 114-281) crystallized
readily and was found to contain three receptors and three ligands
assembled as a hexameric complex in the asymmetric unit. Diffraction
35 data were collected using synchrotron radiation, and the structure was
refined to 2.4 Å resolution and an R-value of 23.2% (R_{free} of 27.7%;
Figure 19). The final model consists of Apo-2L residues 119-131 and
144-281 in each monomer, and Apo-2 residues 21-128 of one copy and 22-
130 of the other two (Figure 20).

40 Each of the three extensive interaction surfaces buries 2750 Å²
of solvent accessible surface area, 1400 Å² from the receptor and 1350

5 Å² from the ligand. Two receptor loops mediate most of the interactions, dividing the interface in two distinct patches (Figure 21): the "50s loop" (residues 51-65; Fig. 1; SEQ ID NO:1) and the "90s loop" (residues 91-104; Fig. 1, SEQ ID NO:1). In patch A, the 90s loop interacts with a cluster of Apo-2L residues around Gln 205 near the bottom of the trimer, while patch B is formed by the 50s loop of Apo-2 and Apo-2L residues clustered around Tyr 216 near the top of the trimer (Figure 21).

15 Patch A is the larger of the two, with 1790 Å² of total buried accessible surface area (880 Å² from the receptor and 910 Å² from the ligand). The 90s loop of Apo-2 contributes 85% of the buried surface area (750 Å²) while the remaining 130 Å² is a result of small contributions from Apo-2 receptor residues 65-69 and 108, 111, 122, and 125.

20 Patch B is the smaller of the two patches (890 Å² total, 480 Å² from the receptor, 410 Å² from the ligand). Here, the 50s loop of the receptor interacts with the 210s and the 150s loops of Apo-2L. This patch is centered on Apo-2L residue Tyr 216, which binds in a hydrophobic groove on the receptor surface formed by the side chains of Apo-2 residues His 53, Asn 55, Leu 57, Leu 58 and Phe 59. The interactions with the 150s loop are more peripheral and polar in nature, and are primarily mediated through contacts between Arg 62 on the receptor with Apo-2L residues Glu 155 and Ser 159. The actual ligand-binding motif found in CRDs 2 and 3 corresponds to Apo-2 residues 43 to 130.

30 * * * * *

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia, USA (ATCC):

35 <u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	209021	May 8, 1997
3F11.39.7	HB-12456	January 13, 1998
3H3.14.5	HB-12534	June 2, 1998
3D5.1.10	HB-12536	June 2, 1998

June 2, 1998

10 This deposit was made under the provisions of the Budapest
Treaty on the International Recognition of the Deposit of Microorganisms
for the Purpose of Patent Procedure and the Regulations thereunder
(Budapest Treaty). This assures maintenance of a viable culture of the
15 deposit for 30 years from the date of deposit. The deposit will be made
available by ATCC under the terms of the Budapest Treaty, and subject to
an agreement between Genentech, Inc. and ATCC, which assures permanent
and unrestricted availability of the progeny of the culture of the
deposit to the public upon issuance of the pertinent U.S. patent or upon
20 laying open to the public of any U.S. or foreign patent application,
whichever comes first, and assures availability of the progeny to one
determined by the U.S. Commissioner of Patents and Trademarks to be
entitled thereto according to 35 USC Section 122 and the Commissioner's
rules pursuant thereto (including 37 CFR Section 1.14 with particular
reference to 886 OG 638).

25 The assignee of the present application has agreed that if a
culture of the materials on deposit should die or be lost or destroyed
when cultivated under suitable conditions, the materials will be
promptly replaced on notification with another of the same.
Availability of the deposited material is not to be construed as a
license to practice the invention in contravention of the rights granted
under the authority of any government in accordance with its patent
laws.

30 The foregoing written specification is considered to be
sufficient to enable one skilled in the art to practice the invention.
The present invention is not to be limited in scope by the construct
deposited, since the deposited embodiment is intended as a single
illustration of certain aspects of the invention and any constructs that
are functionally equivalent are within the scope of this invention. The
35 deposit of material herein does not constitute an admission that the
written description herein contained is inadequate to enable the
practice of any aspect of the invention, including the best mode
thereof, nor is it to be construed as limiting the scope of the claims
to the specific illustrations that it represents. Indeed, various
40 modifications of the invention in addition to those shown and described
herein will become apparent to those skilled in the art from the
foregoing description and fall within the scope of the appended claims.

- 5 What is claimed is:
1. A method of inducing apoptosis in mammalian cells expressing Apo-2 receptor comprising exposing mammalian cells expressing Apo-2 receptor to an effective amount of an Apo-2 agonist antibody.
- 10 2. The method of claim 1 wherein said Apo-2 agonist antibody is a monoclonal antibody.
3. The method of claim 1 wherein said Apo-2 agonist antibody is a chimeric antibody.
- 15 4. The method of claim 1 wherein said Apo-2 agonist antibody is a humanized antibody.
- 20 5. The method of claim 1 wherein said Apo-2 agonist antibody is a human antibody.
- 25 6. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12456.
- 30 7. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12534.
8. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12536.
9. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12535.
- 35 10. A method of treating cancer, comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist antibody.
11. The method of claim 10, wherein said cancer cells are lung cancer cells.
- 40 12. The method of claim 10, wherein said cancer cells are colon cancer cells.

5 13. The method of claim 10, wherein said cancer cells are glioma cells.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

665T60" OT 29666 Fig. 1

1 CCCACGGCGT CCGATAAATC AGCAGCGGGC CGGAGAAACC CGCATCTCT CGCCCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAC
GGGTCCGAG CCGTATTAG TCGTCCGCGG GCCTCTTGGG GCGTTAGAG GCGGGTGT TATGTGGCT GCTACGGGCT AGATGAAATT CCGGACTTTG

101 CCACGGGCGT GAGAGACTAT AAGAGCGTTC CCTACCGCCA TGGACAAAC GGCACAGAAC GCGCGGCGG CTTCCGGGGC CCGGAAAAG CACGGCCCCAG
GGTCCCGGA CTCTCTGATA TTCTCGCARG GGATGGCGGT ACCTTGTTGC CCCTGTCTTG CCGGGCCGGC GAAGCCCCCG GGCCTTTTC GTCCGGGGTC

1 MetGluGlnAsp gclglnasn AlaProAla laSerGlyAl aArgLysArg HisGlyProGly

201 GACCCAGGGA GGGCGGGGA GCCAGGCCGT GGTCGGGGT CCCCAGACC CTTGTGCTCG TGTGCGCGG GGTCTGCTG TTGCTCTCAG CTGAGTCTGC
CTGGGTCCCT CCGGCCCT CCGTCCGAC CCGAGGCCA GGGTTCTTG GAACAGAGC AACACGGCG CCAGGACGAC AACACAGTC GACTCAGACG

22 ProArgG1 uAlaArgGly AlaArgProG lYLeuArgVa lProLysThr LeuValLeuV alValAlaAl aValLeuLeu LeuValserA laGluSerAla

301 TCTGATCACC CAACAGACC TAGTCCCCA GCAGAGAGCG GCCCACAA AAGAGAGGTC CAGCCCCCA GAGGATTGT GTCCACCCTG ACACCATATC
AGACTAGTG GTTCTCTG ATCGAGGGT CGTCTCTCG CCGGGTGTG TTTCTCTCAG GTCCGGGAGT CTCCCTAACA CAGGTGGACC TGTGTATAG

55 LeuIleThr GlnGlnAspL euAlaProG1 nGlnArgAla AlaProGlnG lNlysArgSe rSerProSer GluGlyLeuC ysProProG1 yHisHisIle

401 TCAGAGACG GTAGAGATTG CATCTCTG CATCTCTG CATCTCTG TCTCTATATC TCTCTATATC TACTGGAG AAGAGACGAA CGCGACGTG TCCACACTAA
AGTCTTCTG CATCTCTG GTAGAGACG GGTCTCCCTA CCAGTTCAC GTCACGCTTC TCCGTGGA GGCCTTCTT CTAAGAGGAC TCTACACGGC

88 SerGluAspG lYArgAspCy sIleSerCys lYsTyrglyC nThrValCys GlnCysGluG lGlyThrPh eArgGluGlu AspSerProG lMetCysArg

501 CAGGTGAAGT GGAGCTAGT CCGTCCACCA CGACAGAAA CACAGTGTGT CAGTCCGAA GAGGCACCTT CCGGGAAGAA GATTCTCTG AGATGTGCCG
GTCCACTCA CCTCGATTCA GGCACCTGT GGTCTCCCTA CCAGTTCAC GTCACGCTTC TCCGTGGA GGCCTTCTT CTAAGAGGAC TCTACACGGC

122 GlyGluVa lGluLeuSer ProCysThrT hrThrArgAs nThrValCys GlnCysGluG lGlyThrPh eArgGluGlu AspSerProG lMetCysArg

601 GAAGTCCCGC ACAGGGTGT CCAGAGGGAT GGTCAAGGTC GGTGATTGTA CACCTTCGAG TGACATCGAA TGTGTCCACA AAGAAACAGG CATCATCATA
CTTCACGGG GTGCCACAC GGTCTCCCTA CCAGTTCAC GTCACGCTTC TCCGTGGA GGCCTTCTT CTAAGAGGAC TCTACACGGC

155 LysCysArg ThrGlyCysP roArgGlyMe tValLysVal GlyAspCysT hrProThrPse rAspIleGlu CysValHisL yGluSerG1 yIleIleIle

701 GGAGTCACAG TTGCAGCCGT AGTCTTGA TTGTCTGCAA GTCCTTACTG TGGAGAAAG TCCTTCTCTA CCGTAAAGGC ATCTGTCTCAG
CCTCAGTGT AACGTGGCA TCAGAACTAA CACCGACACA CACAAACGTT CAGAAATGAC ACCTTCTTTC AGGAAGGAAT GGACTTCTCG TAGACGAGTC

188 GlyValThrV alAlaAlaVa lValLeuIle ValAlaValP heValCysLy sSerLeuLeu TrpLysLysV alLeuProTy rLeuLysGly lIleCysSerGly

801 GTGGTGGTGG GGACCCCTG CGTGTGACA GAAGCTACA ACGACCTGG GCTGAGGACA ATGCTCTCAA TGAGATCGTG AGTATCTTGC AGCCACCCCA
CACACCCACC CCTGGGACTC GCACACCTGT CTTGAGTGT TGTGGACCC CGACTCTCTG TACAGGAGTT ACTCTAGCAC TCATGAAACG TCGGGTGGGT

222 GlyGlyG1 YAspProGlu ArgValAspA rgSerSerG1 nArgProGly AlaGluAspA snValLeuAs nGluIleVal SerIleLeuG lNProThrGln

901 GGTCCCTGAG CAGGAATAG AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCCCGG GAGTCAGAGC ATCTGCTGGA ACCGGCAGAA
CCAGGGACTC GTCTTTACC TTCAGGTCTT CCGTCTCTC GGTGTCCAC AGTTGTACAA CAGGGGGCC CTGAGTCTCG TAGACGACCT TGGCCGTCTT

255 ValProGlu GlnGluMetG lValGlnG1 uProAlaGlu ProThrGlyV alAsnMetIe userProGly GluSerGluH isLeuLeuG lUpRoAlaGlu

1001 GCTGAAAGGT CTCAGAGGAG GAGGCTGCTG GTTCCAGCAA ATGAAGGTGA TGCCACTGAG ACTCTGAGAC AGTCTTCTGA TGACTTTGCA GACTTGGTGC
CGACTTTCCA GAGTCTCTC CTCGACGAC CAGGTCGTT TACTTCCACT AGGGTGACTC TCAGACTCTG TCACGAAGCT ACTGAACGT CTGAACACG

288 AlaGluArgS erGlnArgA rgArgLeuLeu ValProAlaA snGluGlyAs pProThrGlu ThrLeuArgG lNcysPheAs pAspPheAla AspLeuValPro

1101 CCTTTGACTC CTGGGAGCGG CTCATGAGGA AGTTGGGGCT CATGGACAAAT GAGATAAAGG TGACTAAGG GGCACAGGG ACACCTTGT
GAAA CTGAG GACCTCGG GAGTACTCCT TCACCCCGGA GTACCTGTTA CTCTATTTC ACGATTTC AGUAlaAla GlyHieArga sPThrLeuTy
322 pheAsp8 rTyrpAluPro LeuMetArgL yLeuGlyLe uMetAspRan GluLeuLyv alAlaLyvAl aGluAlaAla
1201 CAGCATGCTG ATAAAGTGG TCAACAAAAC CGGCGAGAT GCTCTGTCC ACACCTGCT GATGCTTG GAGAAGCTG GAGAGGACT TGCCAAAGCAG
GAGTACGAC TATTCACCC ACTTGTTTG CCCGCTCTA CGGAGACAG CGGAGACGA CTCTGCGACC CTCTCTCTGA ACGTTGCTC
355 ThrMetLeu IleLySTpV alaSnLySTh rGlyArgasp AlaSerValH iethrLeuLe uAspAlaLeu GluThrLeuG lyGluArgLe uAlaLySGln
1301 AAGATTGAGG ACCACTTCTT GAGCTCTGGA AAGTTCAAT ATCTAGRAGG TAATGCAGAC TCTGCCWGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA
TCTAACTCC TGGTGACAA CTGAGACCT TTCAAGTACA TAGATCTTCC ATTACGCTG AGACGGAACA GGATTTCACAC TAAGAGAACT CATTCACTCT
388 LybIleGluA sPHisLeuLe uSerSerGly LySPheMetT yrLeuGluG1 yAsnAlaAsp SerAlaXqS erOG*
1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAACTCTC
GGAAGGGACC AAATGGA AAAACCTTTT TCGGGTGCAC CTGAGGTCAG TCATCTCTTC ACGTGTTAA CAGTGACTG GCCATGACCT TCCTTGAGAG
1501 CCATCCAAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACCTGAC TTGGCATTAAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAT
GGTAGGTGT AGTGGGTGAC CTACCTTCTA GGACATTGAA AAGTGACCTG AACCGTAATA AAAATATTTC ACTTACACTA TTATTCTCTGT GATACCTTTA
1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTC AGATTGTTT TGGGATGTCA TTGTTTTCAC AGCACTTTT TATCCTAATG TAAATGCTTT ATTATTAT
CGACCTAGT AAGGCAACA CGCATGAAAC TCTAAACCAA ACCCTACACT AACRAAAGTG TCGTAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA
1701 TTGGGGTACA TTGTRAGATC CATCTACAAA ARAAAAAA ARAAAAAA GCGCGCGCGG ACTCTAGAGT CGACCTGCG AGGCTTGGCC GCCATGGCC
AACCCGATGT AACATTCTAG GTAGATGCTT TTTTCTTTT TTTTCTTTT CCGCGCGCGG TGAGATCTCA GCTGGACGTC TTCGAACCGG CGGTACCGG

Fig. 1 (cont.)

ig. 2 A

1 MEORGONAPASGARKRHGPGPREARGARPGRLVVKTLVLAVALLLVSAESALLTQOD
 61 LAPQORAAPQOKRSSPSEGLCPFGHHSIEDGHDICISCKYGODYSTHWNLLFCLETRCD
 121 SGEVELSPCTTTRNTVCOCEGTREEDSPENCRKCRGTGPRGMVKVGDCTPWSDDIECVH
 181 KESGIIIGVTVAARVLIIVAVUCKSLMKVLPYKIGICSGGGGDPERVDRSSQRPGEAD
 241 NVZNEIVSILQPTQVPEQEMEVQEPAEFTGVNMLSPGESEHLLERAEARSQRRLVPA
 301 NEGDPTEITLROCFDDFADLVFDSWEPFLMRKLGMDNEIKVAKAEARAGHRDTLYTMLIKW
 361 VNKTRGDASVHTLLDALETIGERLAKQKIEDHLLSSGKFMYLEGNADSALS

Apo2
 DR4
 Apo3/DR3
 TNFR1
 Fas/Apo1

L I P D S E P L M S M D E K K A K E A A - - G H R U T
 N I P D S D Q L M Q D T K A A A A A A A - - G P G D A
 V M A A A A A A A A A A A A A A A A A A - - F D Q Q
 V V E N P L R M A A A A A A A A A A A A A A A A A A - - C L E E Q
 I G V M T L S Q V G E V E K N V N E A K H E I K N D V O D T A E Q K V

Apo2
 DR4
 Apo3/DR3
 TNFR1
 Fas/Apo1

T A M K R R Q Q P - A - G G A V A A A A A A A A A A A A A A A A A A
 A A M K R R Q Q P - A - G G A V A A A A A A A A A A A A A A A A A A
 V S A
 - Q L R N H Q L H K K E A Y - D E I K D H K K A N C T L A C K A Q T

Fig. 3

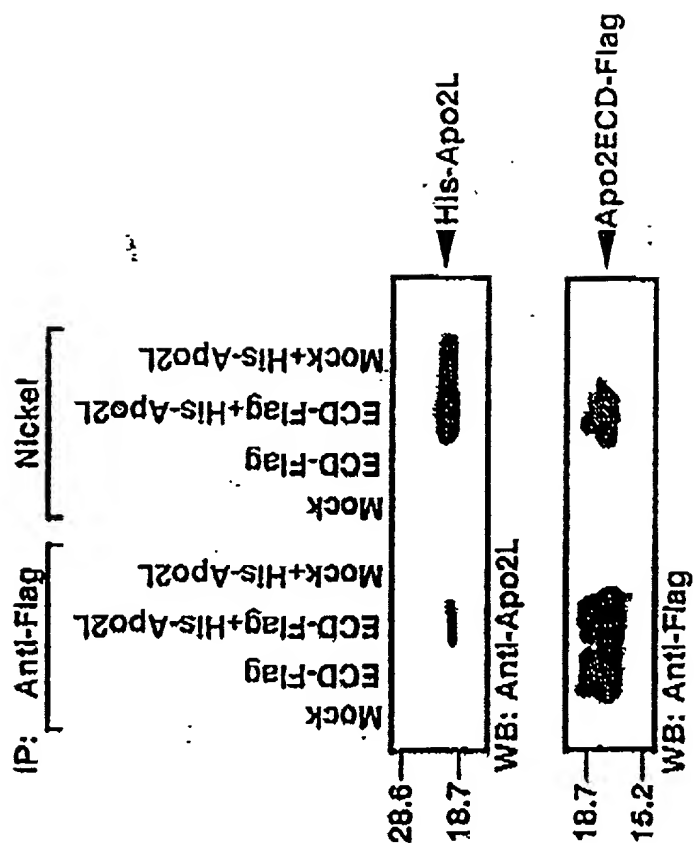


Fig. 4

665T60" 0T 296860

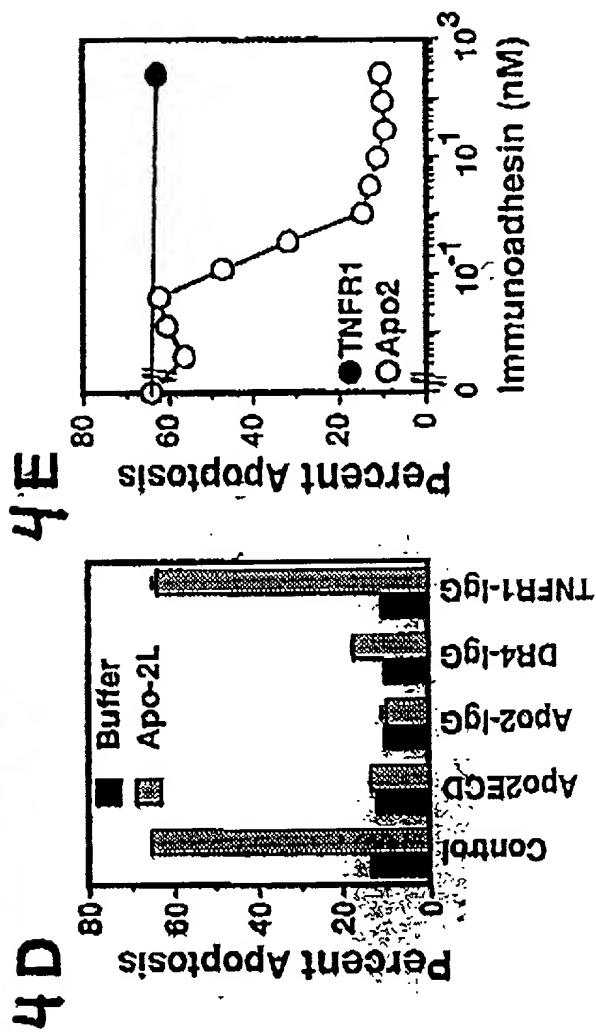
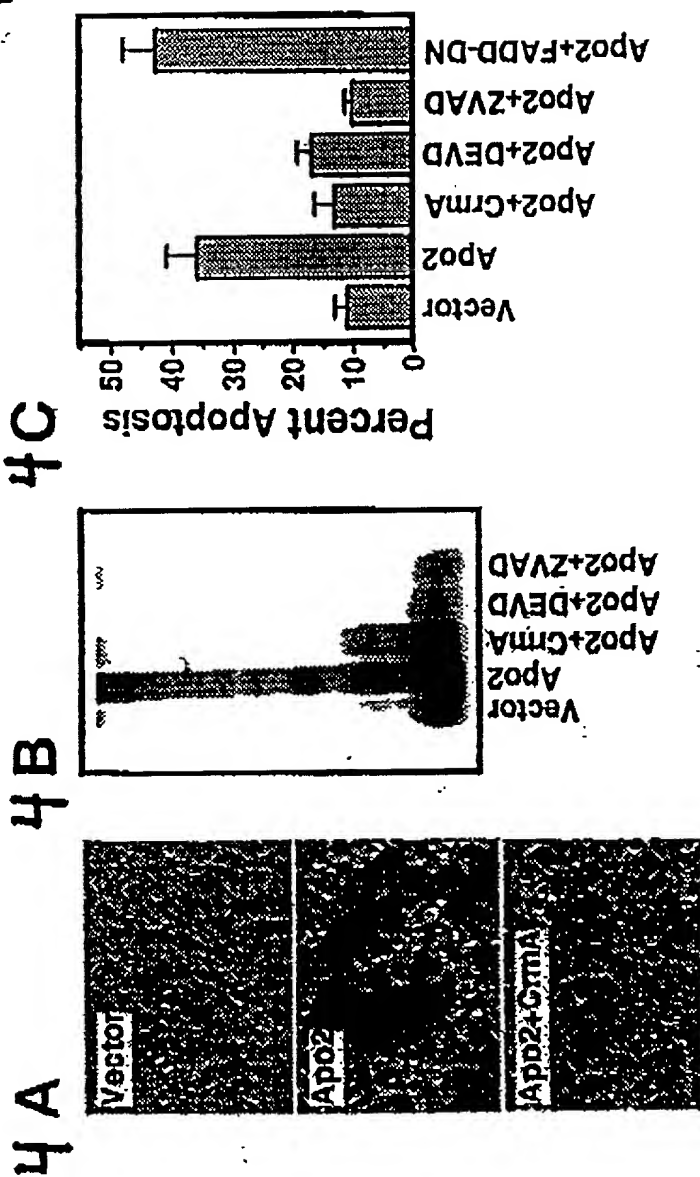
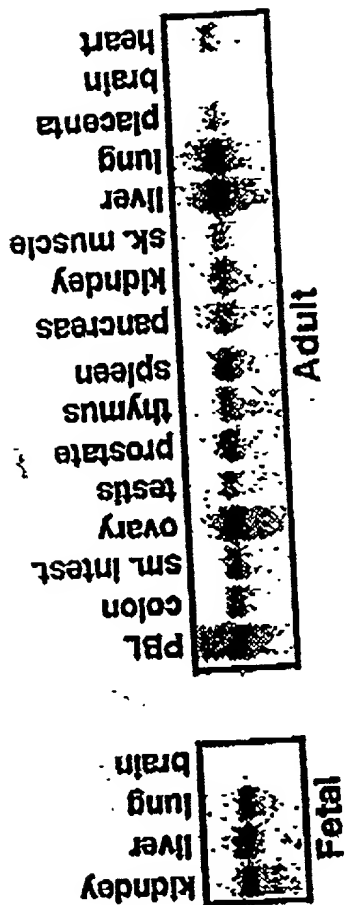


FIG. 6



659T60" 01/29/80

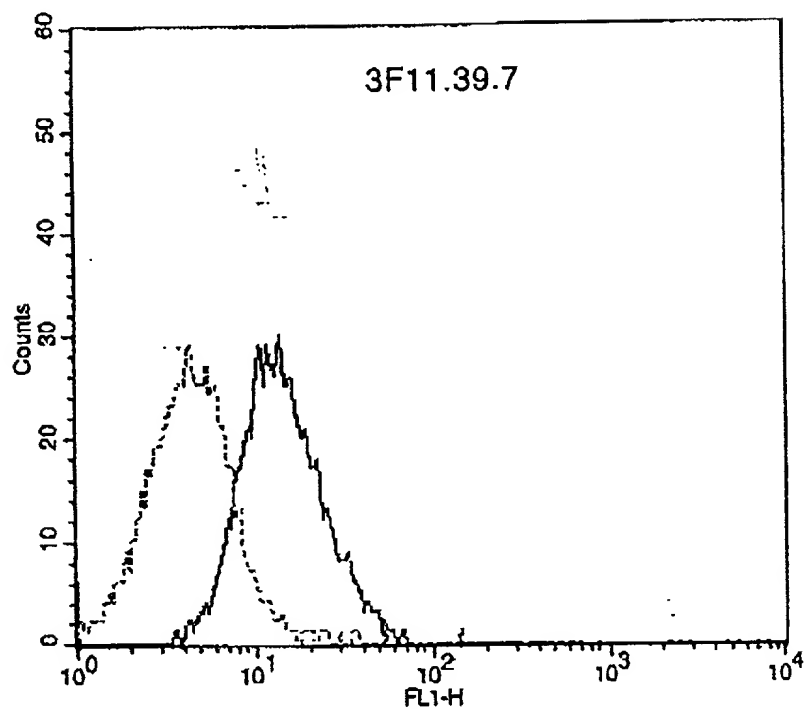


Fig. 7

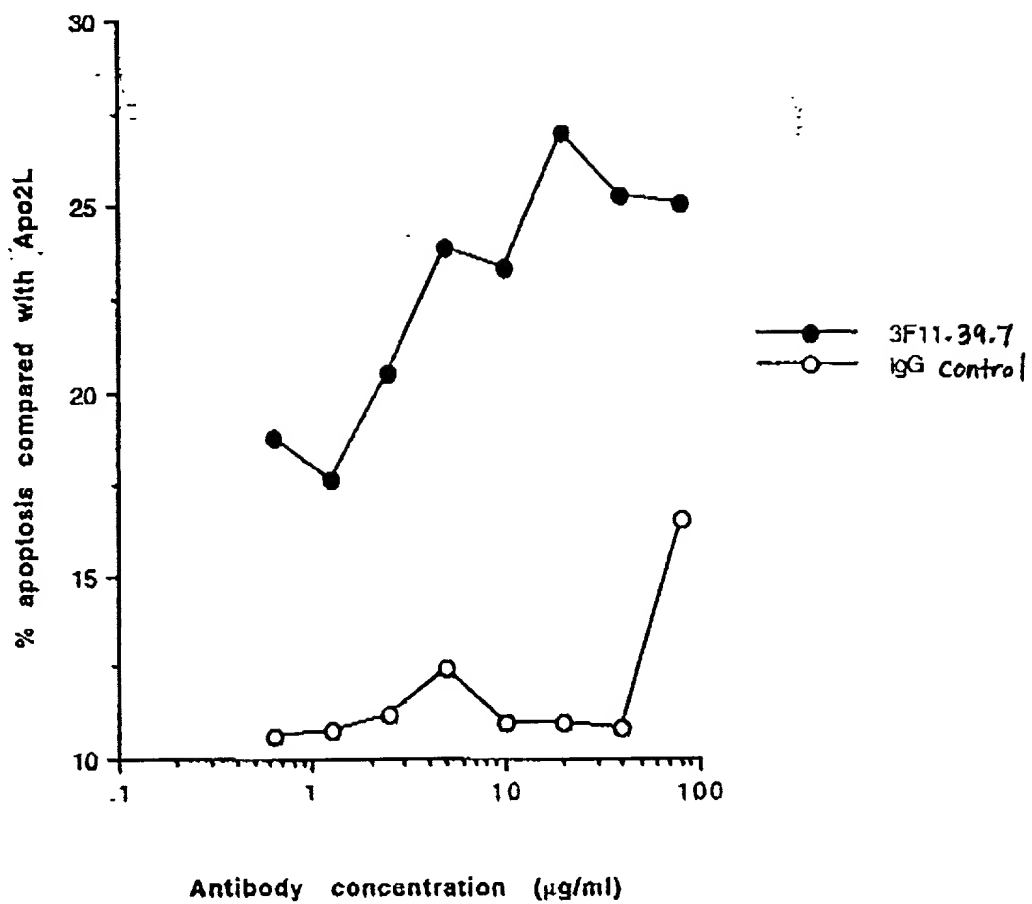


Fig. 8

09363107296660

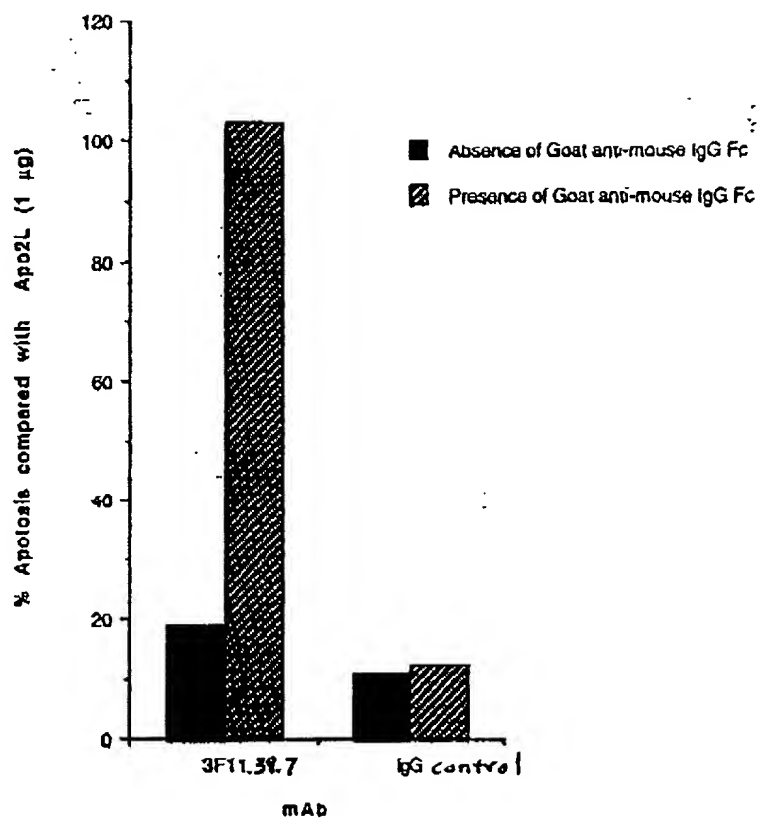


Fig. 9

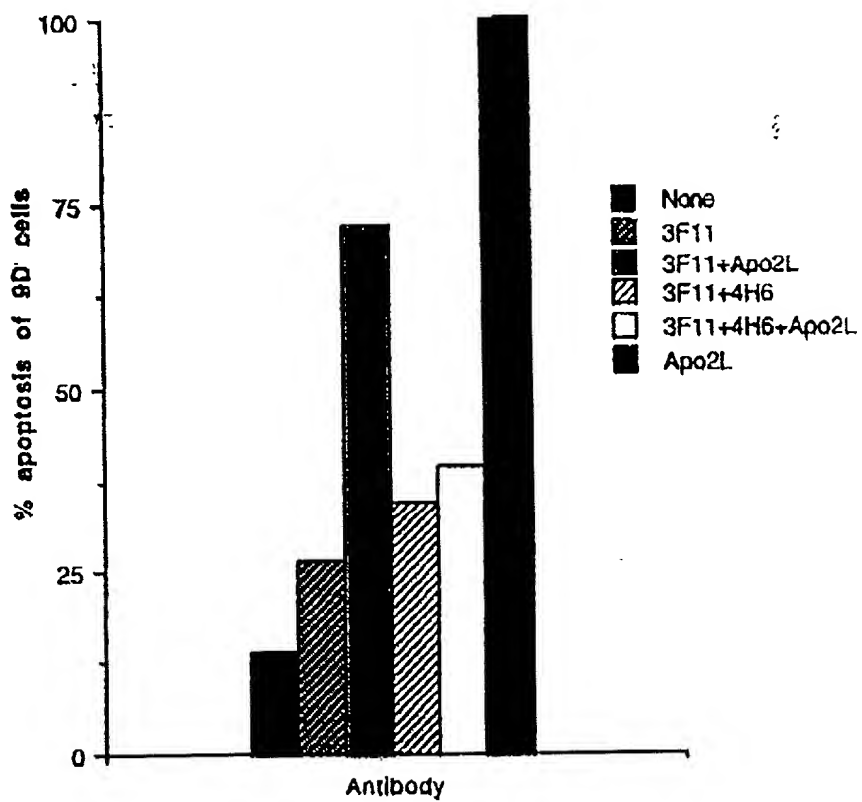


Fig : 10

665T60" 07/29/66

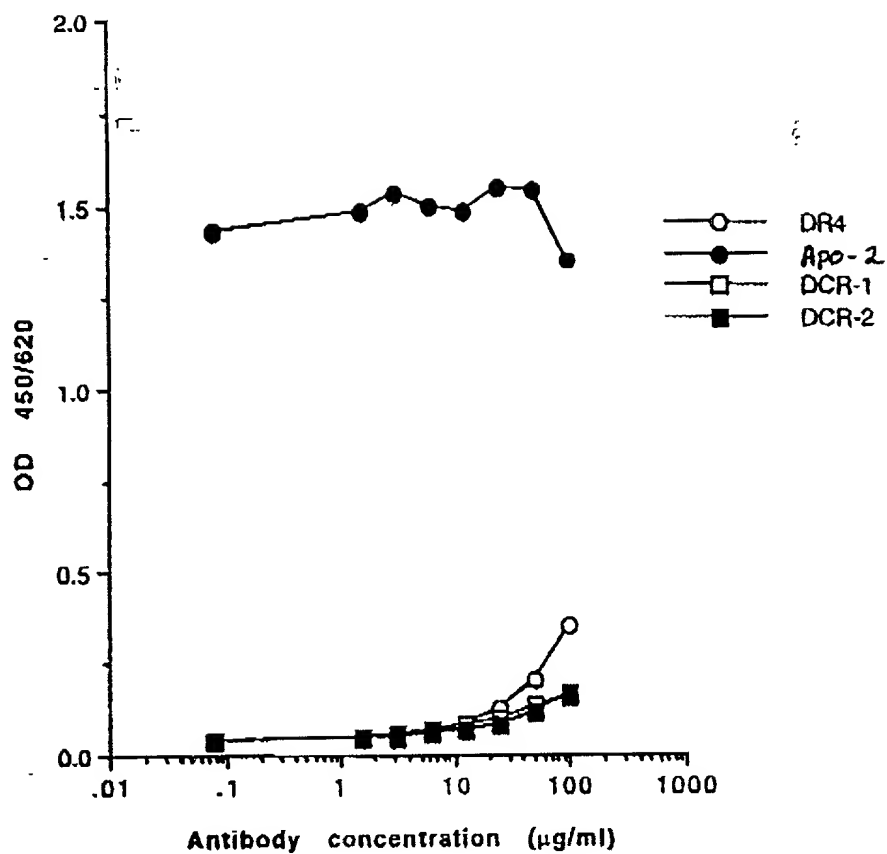


Fig. 11

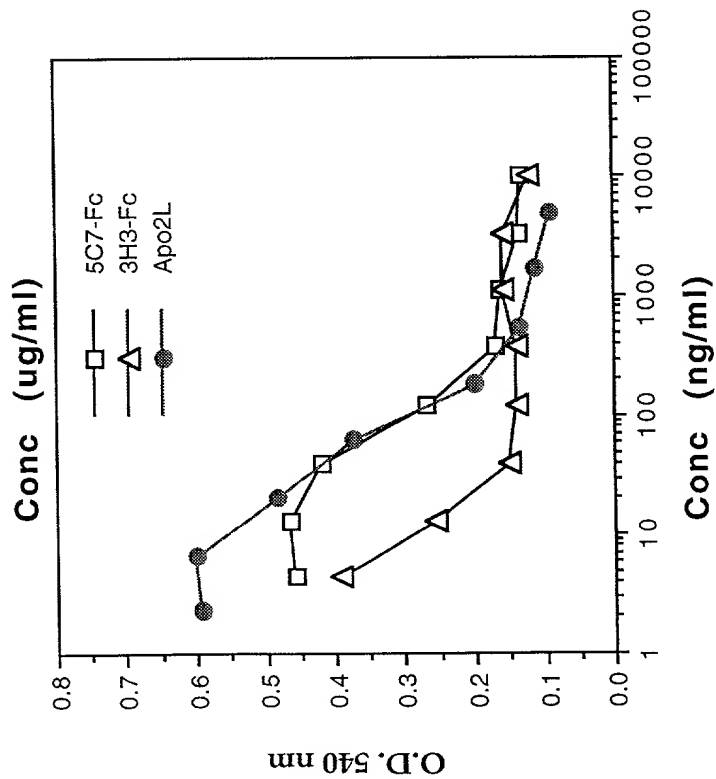
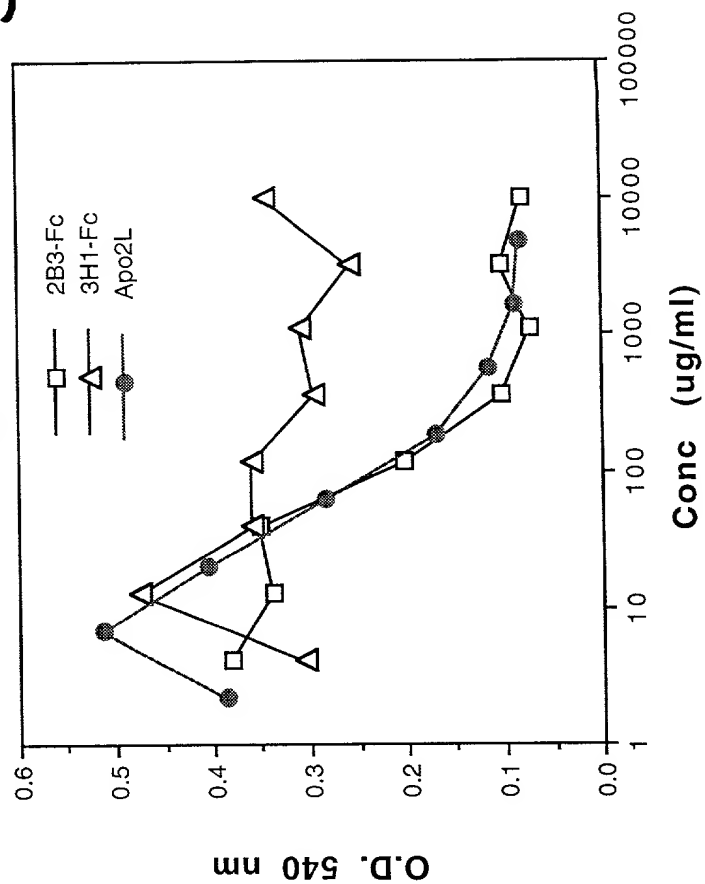
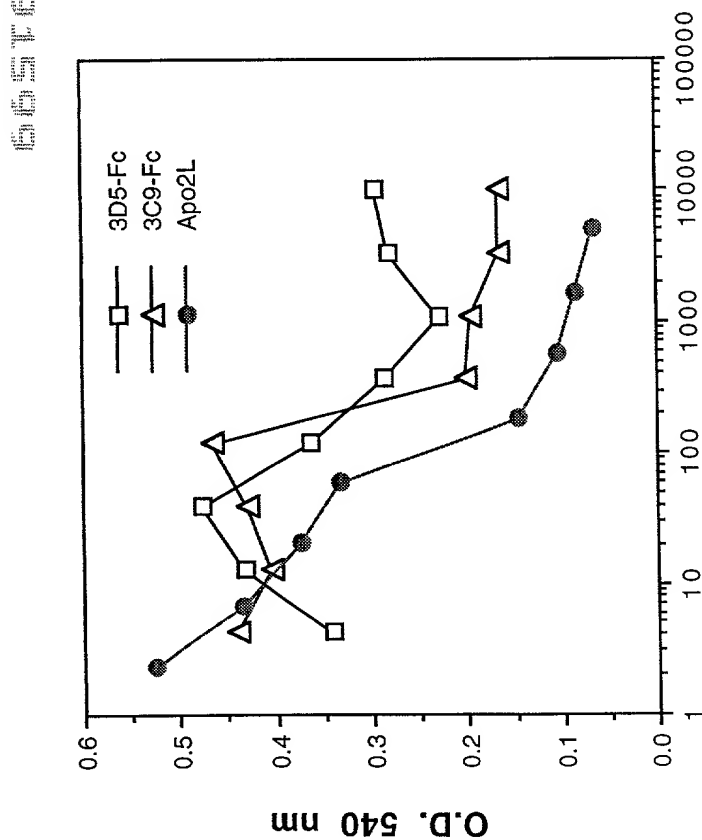
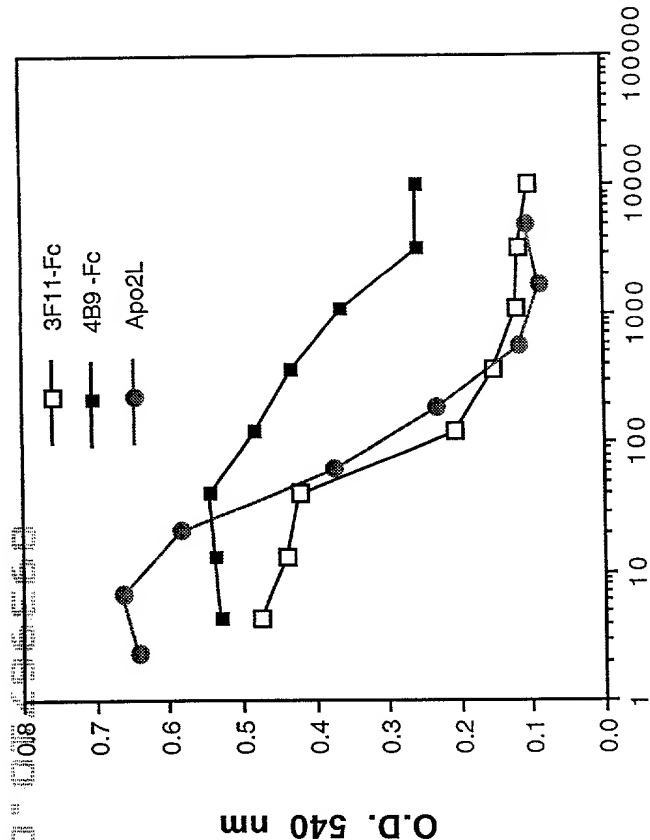
Isotype	Kd-1 (pM)	Bloc	Apop w/L	Cross reactivity			IHC
				DR4	Apo-2	DCR1 DCR2	
2B3.7.1		-	+	+	+++	-	
3F11.39.7	20.	+	+	-	+++	-	+
4B9.23.6		-	+/-	-	+++	-	
5C7.9.1		-	+	+	+++	-	
3H1.18.10		-	-	+	+++	+/-	+
3H3.14.5	4	+	+	+	+++	+/-	+
3D5.1.10		-	+	++	+++	-?	
3C9.8.6		+	+	-	+++	-	
4H10.14.10		+/-	?	+/-	+++	+/-	

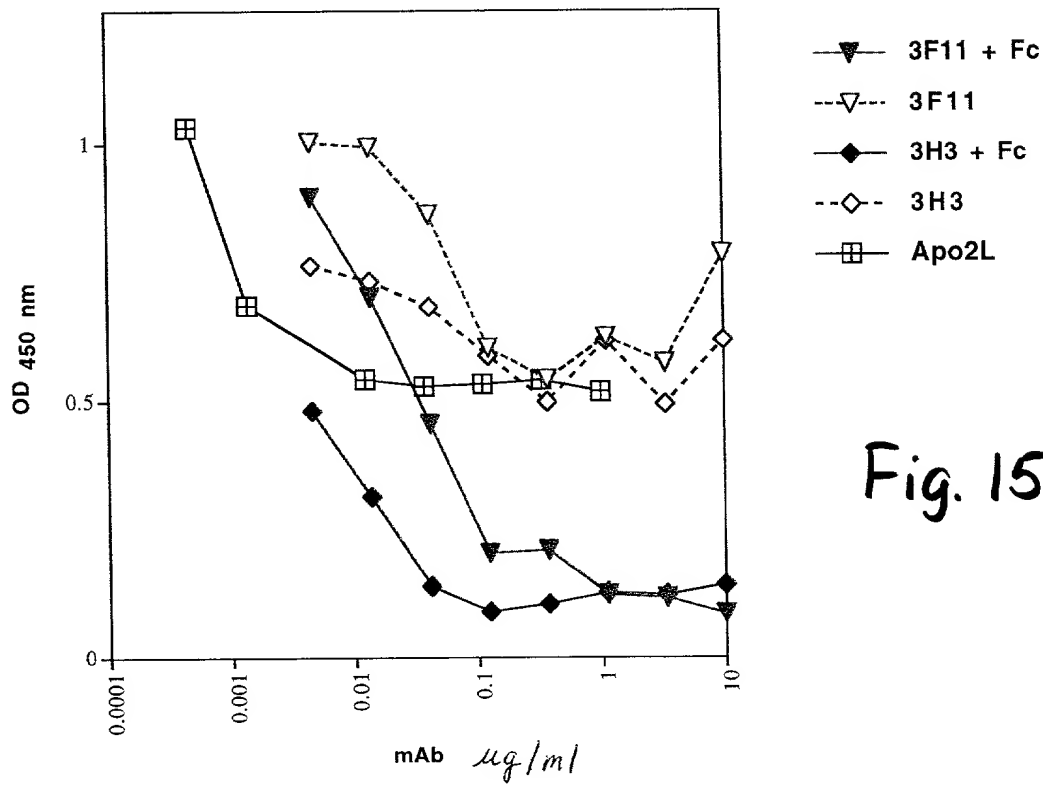
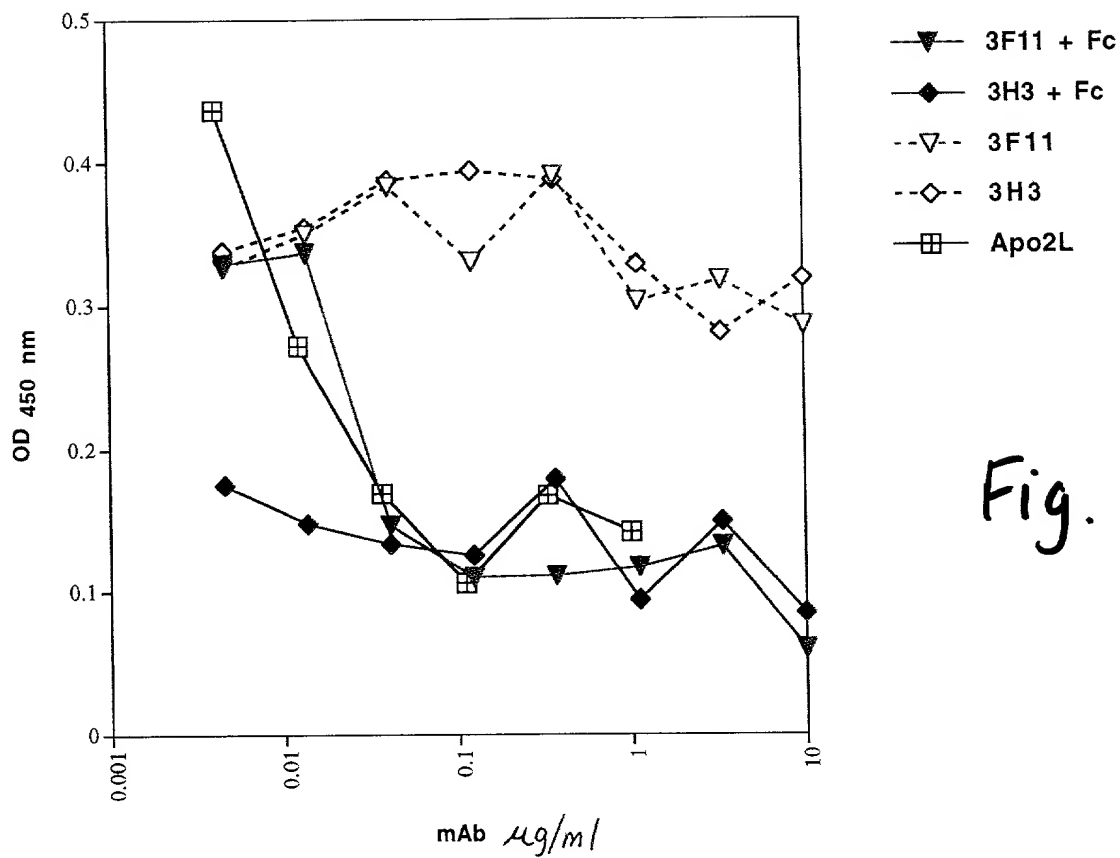
Fig. 12

		Affinity (pM)
DR4-IgG	to Apo2L	82
Apo-2 -IgG	to Apo2L	1
mAb 3F11	to Apo-2 IgG	20
mAb 3H3	to "	3

Fig. 13

Fig. 14





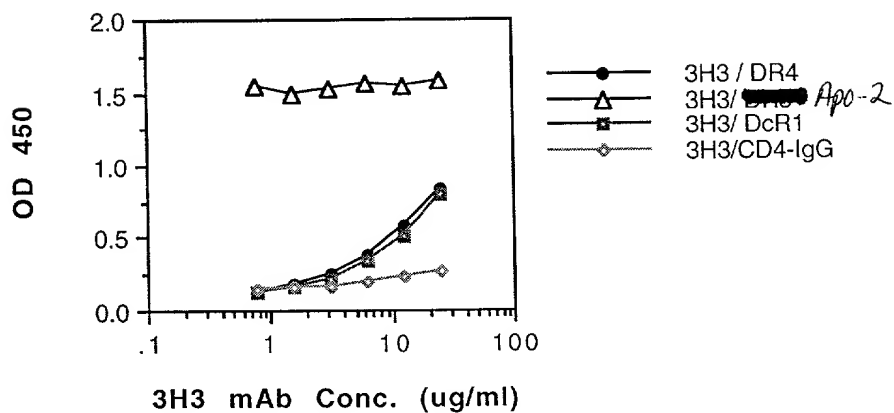
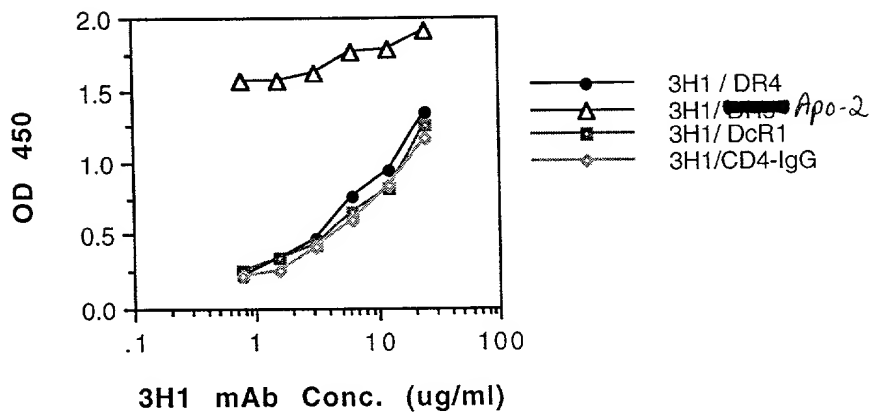
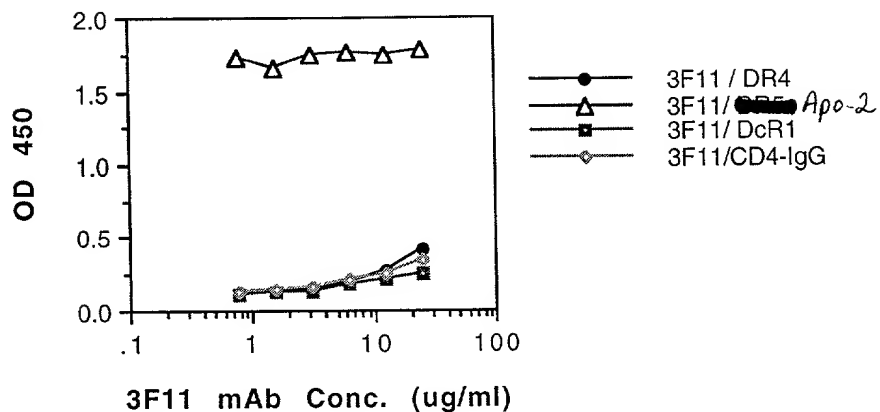
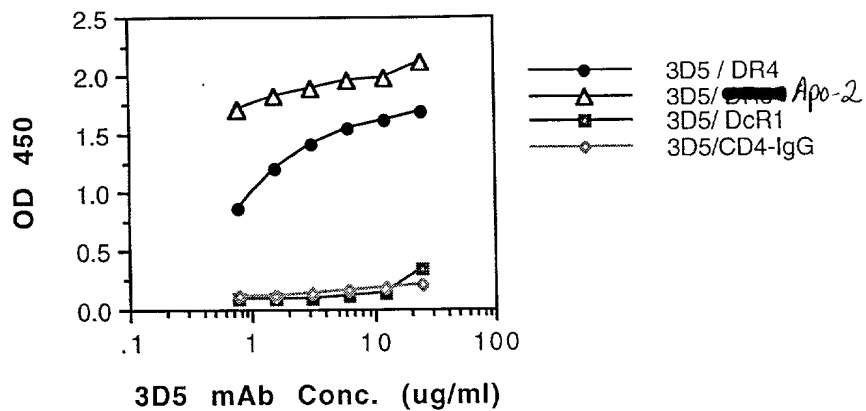


Fig. 16

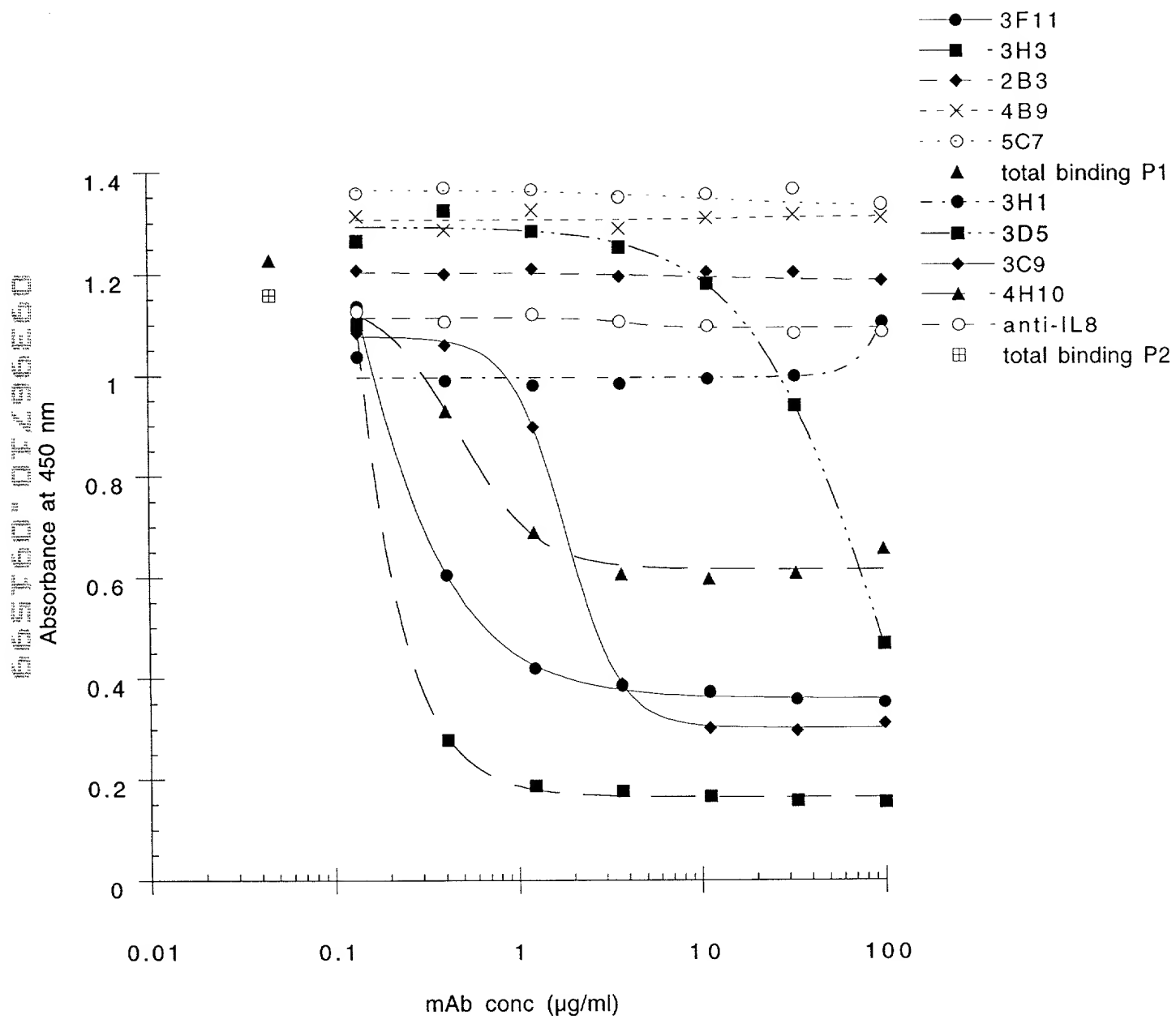


Fig. 17

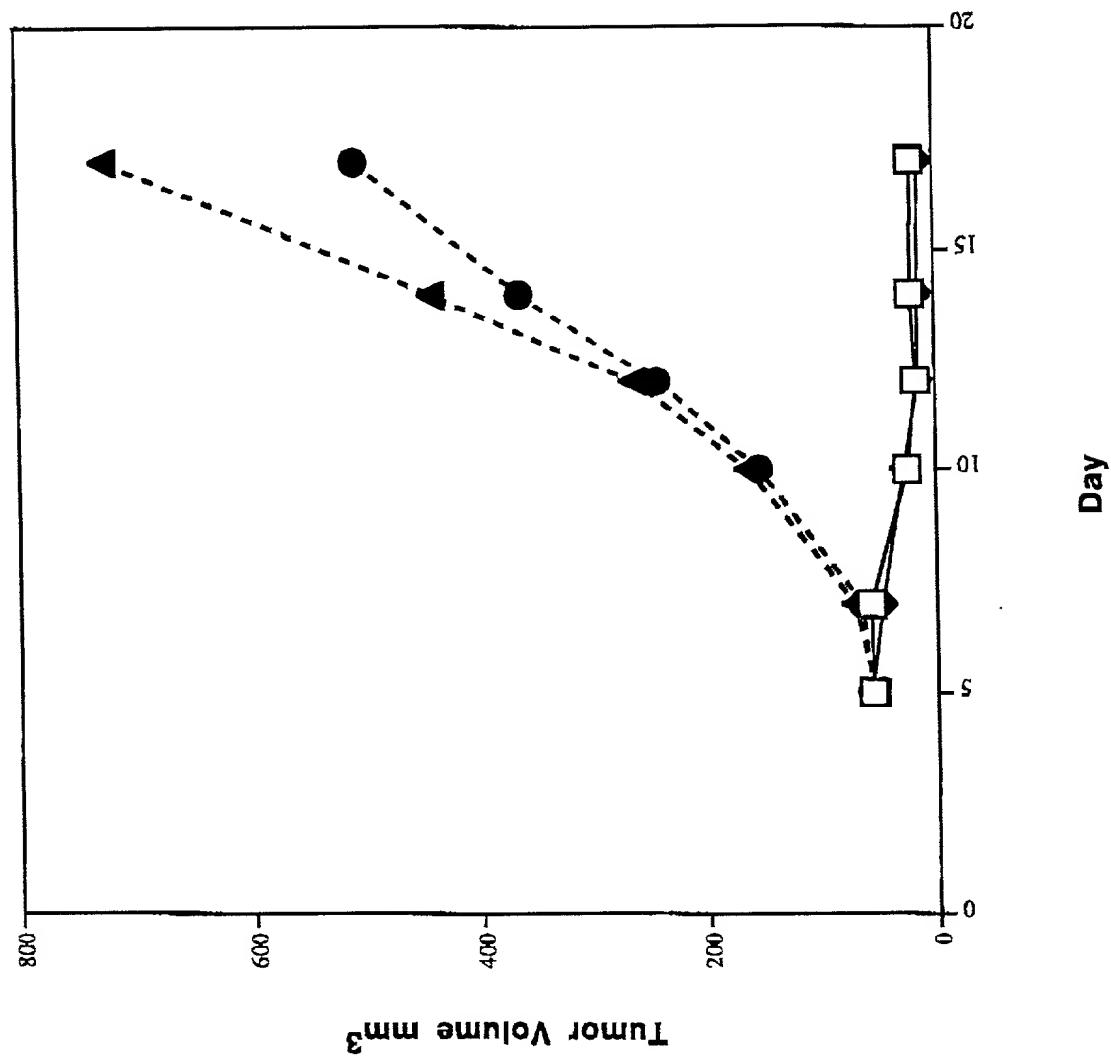


Fig. 18

Data Collection

	In-house	SSRL
Resolution (Å)	30–3.5 (3.62–3.50) ^a	30–2.4 (2.49–2.40) ^a
R _{sym} ^b	0.185 (0.398) ^a	0.056 (0.396) ^a
Number of observations	51,527	152,986
Unique reflections	12,459	38,908
Completeness (%)	99.8 (99.9) ^a	99.8 (99.7) ^a

Refinement

Resolution (Å)	30–2.4
Number of reflections	38,850
Final R ^c , R _{free} (F>0)	0.222, 0.267
Number of residues	781
Number of solvent molecules	286
Number of non-H atoms	6577
Average B factor (Å ²)	47.6
Rmsd bonds (Å)	0.013
Rmsd angles (°)	1.7
Rmsd B (bonded atoms) (Å ²)	2.4

^a Numbers in parentheses refer to the highest resolution shell.

^b $R_{\text{sym}} = \sum ||I - \langle I \rangle| / \sum I$. $\langle I \rangle$ is the average intensity of symmetry related observations of a unique reflection.

^c $R = \sum |F_o - F_c| / \sum F_o$. R_{free} is calculated as R, but for 10% of the reflections excluded from all refinement.

Fig. 19

665T60" 0T/96E60

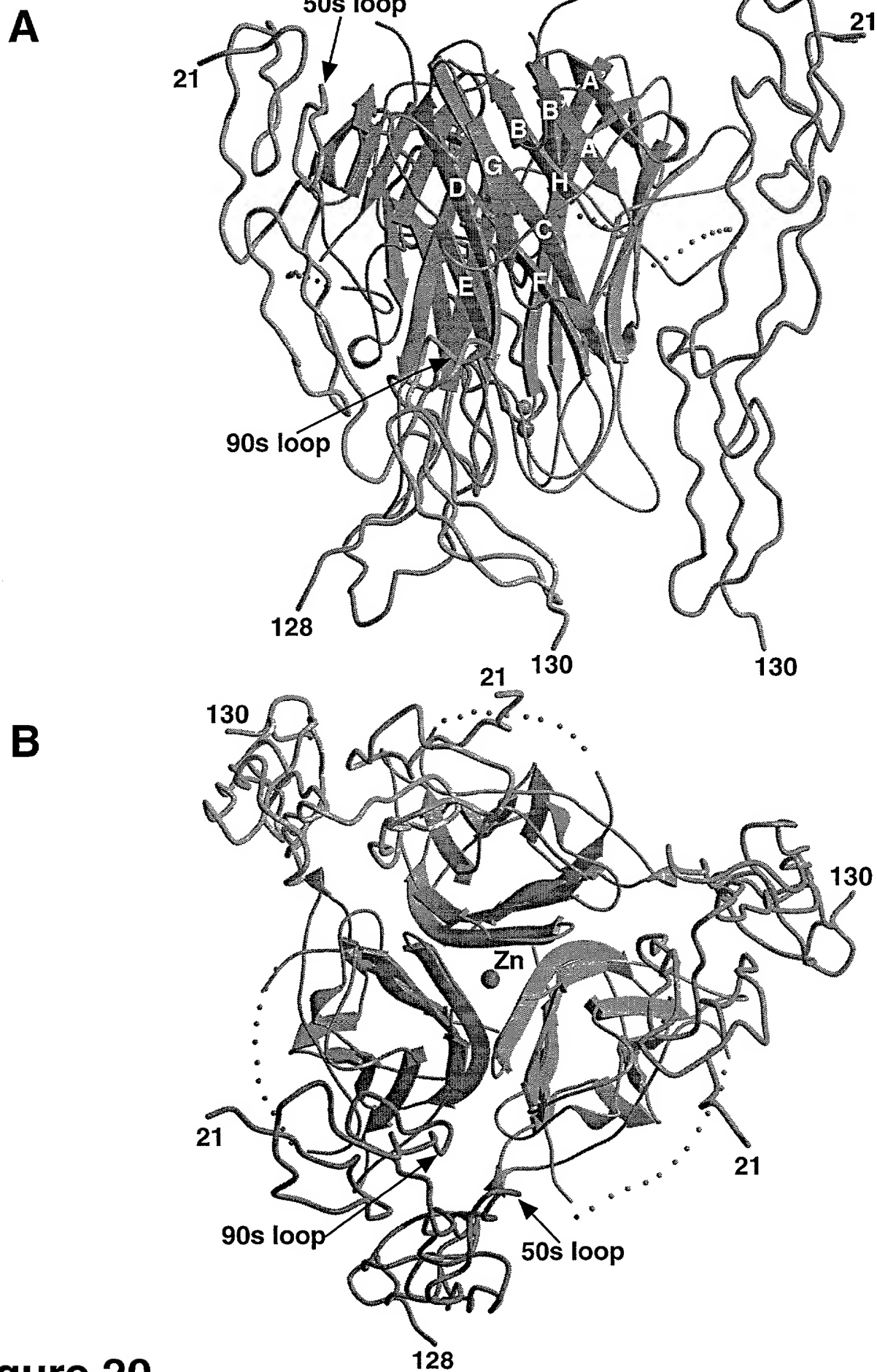


Figure 20

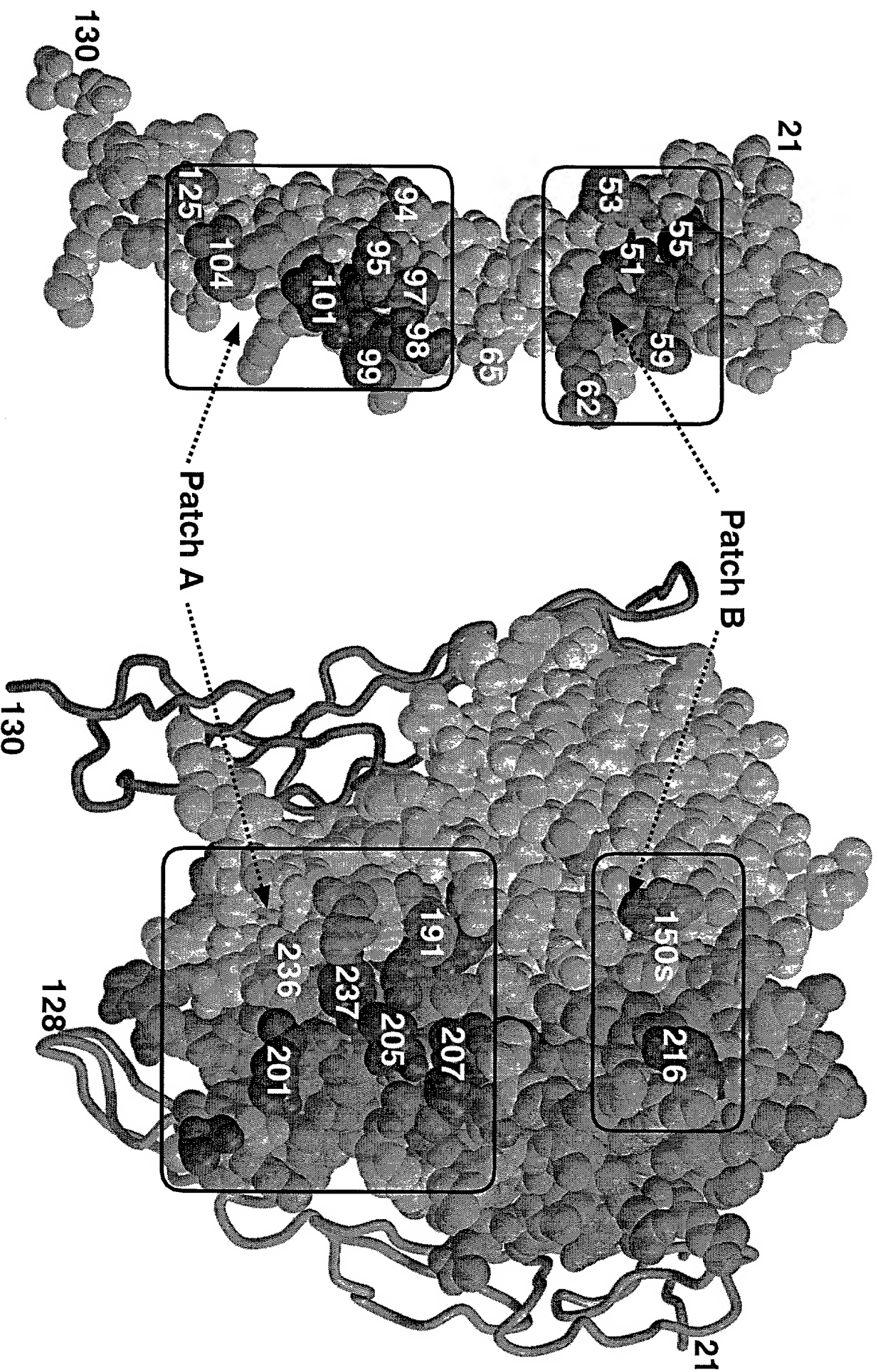


Figure 21